Advances in the diagnosis and epidemiology of *Orthopoxvirus* infection in non-domestic animals and its reservoir hosts in the United Kingdom

Thesis submitted in accordance with the requirements of the

University of Liverpool for the degree of Master in Philosophy

by

Taiana Pereira da Costa

June 2021

Taiana Pereira da Costa, ADVANCES IN THE DIAGNOSIS AND EPIDEMIOLOGY OF *ORTHOPOXVIRUS* INFECTION IN NON-DOMESTIC ANIMALS AND ITS RESERVOIR HOSTS IN THE UNITED KINGDOM

Abstract

Outbreaks of infectious diseases sporadically occur in zoological collections, and many of them are associated with rodents, including cowpox and toxoplasmosis. The incidence of cowpox in both humans and animals in Europe has risen in recent years, leading cowpox virus (CPXV) to be considered an emerging public health threat. The aim of this research project was to contribute to the diagnosis and to a better understanding of the epidemiology of cowpox virus infection in non-domestic animals and its wild reservoirs, and this was achieved in multiple ways. Throughout this text, 'wild' animals refers to free-ranging animals; 'non-domestic' animals refers to non-domesticated animals kept in captivity (e.g. zoological collections); and 'domestic animals' refers to domesticated animals kept in captivity, either as pets (e.g. dogs, cats, horses) or production animals (e.g. cattle).

An immunohistochemical protocol to identify *Orthopoxvirus* (OPV) antigen in formalin-fixed paraffin embedded tissues was developed. This technique was shown to work successfully in tissues from multiple non-domestic species, as well as domestic cat, contributing to a more accurate diagnosis of OPV infections in domestic and non-domestic animals.

This study compiled epidemiological information of previously unpublished cases of OPV infections in non-domestic animals in the United Kingdom, and reported for the first time CPXV in snow leopard, Chilean pudus, aardvark, and Malayan tapir. These results indicate an even wider and more varied range of non-domestic animals susceptible to OPV and CPXV than those previously reported, contributing to a better understanding of the epidemiology of OPV, particularly the wide range of species susceptible to this infection.

The prevalence of OPV and toxoplasmosis in wild small mammals from Chester Zoo was investigated. Results showed that there is strong evidence that OPV and *T. gondii* circulate in wild small mammals in Chester Zoo. These findings highlight the importance of stringent biosecurity measures and pest management control in zoological collections, in order to prevent or reduce the chances of CPXV and toxoplasmosis transmission between wild small mammals and zoo animals or humans occurring.

Taken together, the results of this study contribute to a better understanding of the pathogenesis and epidemiology of cowpox in non-domestic animal species and its reservoir hosts in the United Kingdom and helps to achieve a higher degree

of animal healthcare and welfare in zoological collections. Moreover, this study provides tools and baseline data that can benefit future diagnostic and research trials with non-domestic animals and wild rodents.

Declaration

I declare that the work presented in this thesis is all my own and that it has not been submitted for any other degree.

Acknowledgements

There are a number of people without whom this thesis might not have been written, and to whom I am greatly thankful.

First and foremost, I would like to express the deepest appreciation to my primary supervisor, Prof. Dr. Julian Chantrey, and my secondary supervisors, Dr. Richard Blundell and Dr. Ranieri Verin, whose guidance, support, and help were essential for the development and completion of this project. I especially thank Prof. Dr. Julian Chantrey for his trust, invaluable assistance, encouragement, and mentorship throughout this investigation. I also specially thank my IPAP members, Dr. Gail Leeming and Prof. James Stewart for their guidance and support.

I would like to thank the North of England Zoological Society for providing financial support for this investigation. It was a honour to be a conservation scholar in such a renowned institution. I am truly thankful to Dr. Javier Lopez and Mr. Tim Rowlands, for their help on the design and planning of the project, and to all the staff at Chester Zoo that were involved in the rodent trapping. I also would like to thank Dr. Mark Stidworthy and Dr. Daniela Denk from the International Zoo Veterinary Group for their invaluable collaboration for this project.

I am grateful to all the staff of the Veterinary Pathology Diagnostic Service of the University of Liverpool, for their immense help on processing samples, particularly Valerie Tilston from the histopathology service, Marion Pope from the ultrastructural service, and Dr. Dorina Timofte, Dr. Iuliana Maciuca, and Mr. Andy Wattret from the microbiological service.

Loving thanks to my friends who made these years of combined residency and postgraduate program a lot easier, especially Fran Fernandez, Andy Rich, Guido Rocchigiani, Peter Richards and Flavia Zendri.

And last but not least, from the bottom of my heart, I want to thank my family, specially my daughter Luana and my partner Jonathan Kirkham, for their affection, love and encouragement throughout this journey.

Table of Contents

List of Figures

List of Tables

1. INTRODUCTION

As part of their commitment to high animal welfare standards, the World Zoo and Aquarium Animal Welfare Strategy recommends their member organisations to have in place plans to prevent and address animal disease outbreaks, including disease transmission between animals and people (Mellor et al., 2015). Similarly, the United Kingdom's Secretary of State's Standard of Modern Zoo Practice requires that every zoological collection provides animal healthcare, including investigating morbidity and mortality events (DEFRA, 2012). This is achieved by keeping comprehensive records of the results of post-mortem examination and diagnostic testing, which are then regularly reviewed in order to assess and improve husbandry and preventive medical practices (DEFRA, 2012). Moreover, as regulated by the Health and Safety at Work etc. Act 1974 (Parliament of the United Kingdom, 1974), as an employer, zoological collections have a duty of care to protect the health, safety and welfare of their employees and visitors, including protection from zoonotic infectious diseases. Throughout this text, 'wild' animals refers to free-ranging animals; 'non-domestic' animals refers to non-domesticated animals kept in captivity (e.g. zoological collections); and 'domestic animals' refers to domesticated animals kept in captivity, either as pets (e.g. dogs, cats, horses) or production animals (e.g. cattle).

As observed with production and companion animals, collections of non-domestic animals suffer from sporadic outbreaks of infectious diseases (Stidworthy, 2010; Nemat et al., 2015), and the frequency and severity of these outbreaks are directly related to the degree of biosecurity and preventative measures in place. Many of these infectious diseases in zoo animals are associated with wild rodents, including yersiniosis, leptospirosis, rat bite fever, tularaemia, cowpox and toxoplasmosis (Stidworthy, 2010).

Outbreaks of cowpox virus (CPXV) infection in non-domestic animals, domestic animals and humans are reported occasionally in many parts of the world. Although CPXV has been extensively studied, aspects of the epidemiology and pathobiology of this viral infection vary between regions and are not fully understood. Moreover, the incidence of cowpox in both humans and animals in Europe has risen in recent years, leading CPXV to be considered an emerging public health threat (Vorou et al., 2008; Żaba et al., 2017).

CPXV appears to be a generalised multi-systemic pathogen in abnormal hosts; however, we hypothesise that the infection is asymptomatic and the viral replication is primarily enteric in its reservoir hosts. This overall goal of this research project was to contribute to the diagnosis and to a better understanding of the epidemiology

of cowpox infection in non-domestic animals and its wild reservoirs in the United Kingdom (UK).

Specifically, the first objective of this investigation was to improve the diagnostic capabilities of the Veterinary Pathology Diagnostic Service of the University of Liverpool by developing an immunohistochemistry (IHC) protocol to identify *Orthopoxvirus* (OPV) antigen in formalin-fixed paraffin-embedded (FFPE) tissue.

The second specific objective of this study was to compile epidemiological information of clinical OPV infections in non-domestic animals in the United Kingdom, and to contribute to a better understanding of the epidemiology of this viral infection, particularly the wide range of species susceptible to this infection.

The third specific objective of this study was to determine the prevalence of OPV and toxoplasmosis in wild small mammals from a zoological collection, Chester Zoo. This particular zoo was chosen as it has had clinical cases of OPV and toxoplasmosis outbreaks in the past. We were particularly interested in determining the presence of OPV in the intestinal tract and *Toxoplasma gondii* cysts in the brain of wild small mammals, as these are important pieces of information on the epidemiology and transmission of these two pathogens.

By contributing to a better understanding of the epidemiology of OPV infection in non-domestic animals and its wild reservoirs, this study also indirectly helps to achieve a higher degree of animal health care and welfare in zoological collections. Moreover, this study provides tools and baseline data that can benefit future diagnostic and research trials with non-domestic animals and wild rodents.

2. LITERATURE REVIEW

2.1. Historic background and geographic distribution

Cowpox virus is widely known and of great interest, partially because its zoonotic potential, but also because of its historic role in the discovery of the first human vaccine (Jenner, 1789; Chomel, 2014) and the eradication of smallpox (Fenner et al., 1988), the world's first eradicated disease. For almost 150 years after Edward Jenner had published the ‘‘Inquiry” in 1798 (Jenner, 1798), it was generally assumed that the CPXV was the vaccine against smallpox. It was not until 1939 when it was shown that vaccinia, the smallpox vaccine virus, was serologically related but different from the CPXV (Downie, 1939). In the late 1970s and mid-1980s, with the advance of molecular techniques, it was confirmed that CPXV, vaccinia and variola viruses were indeed closely related but distinct viruses (Müller et al., 1978; Esposito and Knight, 1985), later shown

by full genome sequencing (Goebel et al., 1990; Esposito et al., 2006; Carroll et al., 2011; Dabrowski et al., 2013; Franke et al., 2017).

CPXV is distributed throughout Europe (except Ireland) (Chantrey et al., 1999), western Russia, and adjacent areas of Northern and Central Asia. An increasing number of cowpox virus infection cases in humans and animals have been reported in Europe over the past decade (Damon, 2007; Vorou et al., 2008), mainly in the UK (Public Health England, 2019).

2.2. The cowpox virus

Classification: The *Poxviridae* is a family of complex DNA viruses that replicate entirely in the cytoplasm of vertebrate (subfamily *Chordopoxvirinae*) and invertebrate (subfamily *Entomopoxvirinae*) cells (Moss, 2007). The subfamily *Chordopoxvirinae* consists of eight genera: *Orthopoxvirus*, *Parapoxvirus*, *Avipoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, *Suipoxvirus*, *Molluscipoxvirus* and *Yatapoxvirus* (Moss, 2007). The genus *Orthopoxvirus* consists of the following viruses: camelpox, cowpox, ectromelia, monkeypox, racoonpox, skunkpox, Uasin Gishu, vaccinia, variola and volepox (Moss, 2007).

Virion structure: Poxviruses have a genome composed of a single linear double stranded DNA molecule of 130 to 300 kilobasepairs (kbp) with a hairpin loop at each end; and CPXV have the largest genome of all OPV, averaging above 220 kbp (Moss, 2007). In contrast to other double-stranded DNA viruses, poxviruses encode their own DNA replication and transcription machinery and are able to replicate in the cytoplasm of the host cell, avoiding the risk of integration into the host genome (Moss, 2007).

The intracellular mature virions (MV, see 'viral entry and replication cycle' below) of poxviruses are barrel-shaped (approximately 360 X 270 X 250nm). The internal structure of the virion consists of a dumbbell-shaped core, with aggregates of heterogeneous material, known as the lateral bodies, between the concavities of the core, and an outer lipid membrane bilayer (5-6nm thick) (Moss, 2007).

Viral entry and replication cycle: The fusion of poxviruses with cell membrane during entry requires the formation of an entry fusion complex (mediated by 11 viral proteins), and four attachment proteins: A26, A27, H3, and D8 (Chung et al., 1998; Hsiao et al., 1998; Hsiao et al., 1999; Chi-Long et al., 2000; Chiu et al., 2007; Bernard, 2012).

Poxviruses are considered to be unique among all DNA viruses, because their infection cycle is carried out exclusively in the host cytoplasm (Yael et al., 2010). This is only possible because poxviruses encode their own DNA-dependent RNA

polymerase (Grimm et al., 2019). Viral replication involves several stages, detailed elsewhere (Tolonen et al., 2001; Moss, 2007). In summary, the cytoplasmic life cycle is initiated upon virus entry at the plasma membrane in a poorly understood process (Krijnse Locker et al., 2000) that results in the delivery of the viral core into the cytoplasm. The viral core, where the enzymes required for the processes of viral early transcription are packaged during virion assembly, produces a defined set of early mRNAs in which about half of the genome is transcribed (Moss, 1990). The early genes encode for proteins required for the process of cytoplasmic DNA replication. DNA replication initiates the transcription of late genes, which code for proteins that are necessary for the assembly of new virions. Poxviral virion assembly is complex and involves the acquisition of a double-membraned cisterna derived from the smooth endoplasmic reticulum around the core to form the first infectious form of the virus, the intracellular MV (Sodeik et al., 1993). The MV has a single membrane, is released via cell lysis, and it is thought to mediate transmission between host animals (Bernard, 2012). A small percentage of the intracellular MVs become enwrapped by a double membrane cisterna of the *trans*-Golgi network to form the intracellular enveloped virus (EV) (Schmelz et al., 1994), which is capable of polymerising actin tails (Cudmore et al., 1995) and is released via exocytosis (Ichihashi et al., 1971; Morgan, 1976; Payne and Kristenson, 1979), spreading within the host (Bernard, 2012).

Intracytoplasmic viral inclusions: Intracytoplasmic inclusion bodies (ICIBs) are a typical histological feature of OPV infections, and two morphologies have been described. The B-type basophilic inclusion bodies (also known as Guarnieri bodies) are perinuclear viral inclusions, contain the viroplasm and MV particles, and are produced by all poxviruses (Damon, 2007). On the other hand, the A-type eosinophilic (acidophilic) inclusion bodies (also known as Downie bodies) contain MVs clustered within an intracytoplasmic structure, and are only produced by some members of the *Chordopoxvirinae* subfamily, including CPXV (Damon, 2007). A-type inclusions are encoded by the *ati* gene, also named *cpxv158* for the CPXV reference strain Brighton Red, a virus that was originally isolated in 1937 from lesions of the hands of a milk maid and that circulates in Great Britain.

Clinical presentation: The type of disease that results from OPV infections is dependent on several factors, such as viral strain, route of entry, and infectious dose, as well as host species and immune status (Damon, 2007; Essbauer et al., 2010; Hoffmann et al., 2015; Weber et al., 2020). The clinical presentation and histopathological lesions caused by CPXV is usually subtle in the reservoir rodent host species, but can be very severe in naïve host species.

CPXV is epitheliotropic, and CPXV infections often start as vesicular lesions, developing into a pustule with an indented centre and raised erythematous border, which may be followed by secondary bacterial infection. In non-domestic animals, the clinical presentation is rather similar amongst different species and mostly results in localised or multiple lesions on the skin and mucous membranes. Less often, animals show severe respiratory disease without skin lesions or from a generalised rash (Kurth and Nitsche, 2012).

Diagnosis of OPV and CPXV: A presumptive diagnosis of CPXV infection is based on the characteristic clinical presentation, and the histological detection of A-type eosinophilic inclusion bodies in the cytoplasm of infected epithelial cells (Kurth and Nitsche, 2012).

The laboratory diagnosis of OPV infections may be achieved using a combination of serological (e.g. gel precipitation assay, enzyme immunoassay, radioimmunoassay, haemagglutination inhibition test, and neutralisation reaction), morphological (e.g. electron microscopy), biological (e.g. chicken embryo assay and cell culture assay) and biochemical (e.g. polymerase chain reaction [PCR] assays and immunohistochemistry [IHC]) methods (Shchelkunov et al., 2005).

Electron microscopy is a relatively rapid test (1.5-2h), and allows the visualisation of viable and non-viable OPV viral particles. However, except for parapoxviruses, it is not possible to differentiate the species of OPVs or other vertebrate poxviruses using electron microscopy (Shchelkunov et al., 2005).

PCR assays for the detection of OPV DNA have been extensively used (Kurth and Nitsche, 2007; Jeske et al., 2019), but further tests must be used to differentiate between OPV species. These include real-time PCR (Gavrilova et al., 2010; Maksyutov et al., 2015), multiplex real-time PCR (Shchelkunov et al., 2011), restriction fragment length polymorphism (Meyer et al., 1997; Loparev et al., 2001), and sequencing and phylogenetic analysis (Ropp et al., 1995; Chantrey et al., 1999; Cardeti et al., 2011; Dabrowski et al., 2013; Prkno et al., 2017).

Immunohistochemistry is also a useful tool in the diagnosis of OPV. An anti-vaccinia antibody allows the detection of OPV antigen in FFPE tissues (Schulze et al., 2007), while species-specific antibodies are useful to confirm the species of OPV involved. For instance, the use of a cowpox virus-specific monoclonal antibody allows the detection of CPXV antigen in FFPE tissues (Schaudien et al., 2007; Herder et al., 2011).

2.3. CPXV reservoir hosts, maintenance and transmission cycle

A reservoir is defined as “an alternative or passive host or carrier that harbours pathogenic organisms without injury to itself and serves as a source from which other individuals can be infected” (Miller-Keane, 2006). Although a definite source of CPXV infection has only occasionally been identified, different vole species, mice, and gerbils are thought to be the reservoir hosts different parts of Eurasia (Marennikova et al., 1984; Damon, 2007; Weber et al., 2020), and the species of rodent implicated in the maintenance and transmission cycle of CPXV varies accordingly to geographical location (Pastoret et al., 2000; Essbauer et al., 2010; Fischer et al., 2020; Grzybek et al., 2020).

For instance, high prevalence of OPV-reactive antibodies were detected in bank voles (approx. 76%) (*Myodes glareolus*), field voles (approx. 91%) (*Microtus agrestis*) and wood mice (27%) (*Apodemus sylvaticus*) (Crouch et al., 1995; Chantrey et al., 1999) in Great Britain, and in bank voles (71.4%) and striped field mice (66.7%) (*Apodemus agrarius*) in Hungary (Oldal et al., 2015). On the other hand, moderate to low seroprevalence was observed in suslicks (15.3%) *(Spermophilus citellus*) and great gerbils (18.6%) (*Rhombomys opimus*) in Turkmenistan (Ladnyi et al., 1975; Marennikova et al., 1984) and in gerbils (9.2%) *(Meriones libycus*) in Georgia (Tsanava et al., 1989). Low seroprevalence was observed in common voles (0.52%) (*Microtus arvalis*) and bank voles (0.19%) in Germany, while no OPV-reactive antibodies were observed in field voles, wood mice, and striped field mice in Germany (Fischer et al., 2020) or in common voles (*Microtus arvalis*), social voles (*Microtus socialis*), common shrews or wood mice in Georgia (Tsanava et al., 1989). Serosurveillance studies of wild brown rats (*Rattus norvegicus*) have either failed to detect OPV-reactive antibodies (in Great Britain) (Crouch et al., 1995; Webster and MacDonald, 1995) or have detected a very low prevalence (1.7%, in Georgia) (Tsanava et al., 1989), suggesting that rats are unlikely true reservoir hosts of CPXV (Chantrey et al., 1999).

Despite the high numbers of OPV seroprevalence surveys in wild rodents, virus isolation and molecular detection are rarely reported. One study detected CPXV from blood cell pellets of bank voles (4.5%) and wood mice (2.3%) in Great Britain (Chantrey et al., 1999). CPXV has been isolated from a common vole in Germany (Hoffmann et al., 2015), root voles (*Microtus oeconomus*) (L'Vov et al., 1988) and laboratory rats in Russia (Marennikova et al., 1978), from which it spread to zoo animal and humans (Marennikova et al., 1977; Marennikova et al., 1984), and from 0.23% of rodents tested in Turkmenistan (Marennikova et al., 1984).

The transmission of CPXV from the rodent reservoirs to other non-domestic animal species is thought to occur through direct contact, either by bite or scratch, or orally, when rodents are used as a food item (Marennikova et al., 1977; Martina et al., 2006; Kurth et al., 2008; Kurth et al., 2009). In experimentally infected animals, viral shedding occurs via oropharynx in common voles and Wistar rats (Hoffmann et al., 2015), and via urine and faeces in rats (Shchelkunov et al., 2005). Chantrey has occasionally observed A-type eosinophilic intracytoplasmic inclusion bodies in intestinal epithelial cells of wood mice (Chantrey, unpublished data; Figures 2.1). However, evidence is lacking to indicate whether CPXV replicates in the intestinal tract of their reservoir host. If so, the faecal-oral route could also be a potential route of transmission between rodents and non-domestic animals.

2.4. Cowpox virus infection in domestic animals

The domestic cat is the species in which CPXV is most frequently diagnosed in western Europe (Godfrey et al., 2004; Schaudien et al., 2007; Schöniger et al., 2007; Herder et al., 2011; Jungwirth et al., 2018) and, unlike rodents, the reservoir hosts of CPXV, cats are incidental hosts of CPXV and generally develop systemic disease (Bennett, 2014).

Cats probably become infected via skin inoculation or from infected rodent bites (Chantrey et al., 1999; Pastoret et al., 2000), and skin lesions are commonly found on the head, neck and forelimbs. Clinical sign of systemic disease, such as pyrexia, lethargy and/or pneumonia can occur, and is often associated with immune disfunction and death (Hinrichs et al., 1999; McInerney et al., 2016). Moreover, in domestic cats, CPXV can infect and cause disease within the central nervous system, or present solely as a laryngeal mass, without concomitant lesion on the integumentary or respiratory systems (Breheny et al., 2017).

Most infected cats are adults which inhabit rural areas and are known by their owners to hunt wild rodents; furthermore, most cases are seen in the autumn (between August and November), reflecting the population and infection dynamics in rodents (Pastoret et al., 2000). Although cat-to-cat transmission can occur, this is rare. Therefore, cats are not endemic hosts, and serological surveys generally find a low prevalence of infection (Pastoret et al., 2000).

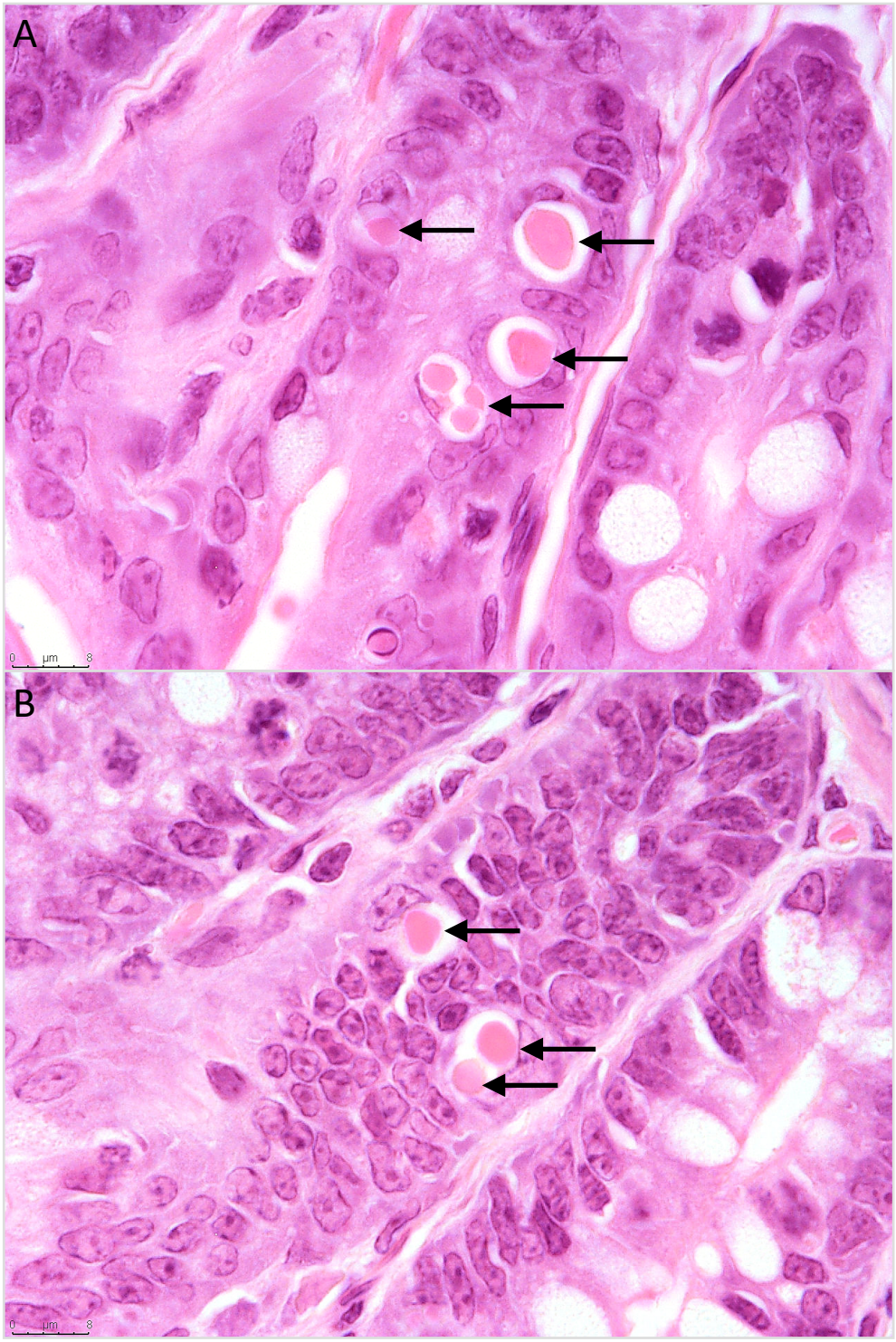

Figure 2.1 - (A and B) Photomicrograph of the small intestine of an adult wood mouse showing intracytoplasmic eosinophilic inclusion bodies (black arrows) in crypt epithelial cells, suggestive of *Orthopoxvirus* infection (100X, scale bar 8µm, HE).

Typically, in both natural and experimental feline infection, a primary skin lesion develops at the site of inoculation within a few days. A viraemia develops, mediated by leucocytes, probably monocytes/macrophages, with virus replication in lymphoid tissue, the lungs and nasal turbinates (Bennett et al., 1989).

Severe disease in cats, e.g. pneumonia or large non-healing skin lesions, is often associated with immunosuppression and has a poor prognosis. This may result from co-infection with feline leukaemia or feline immunodeficiency viruses, or other concurrent diseases (Pastoret et al., 2000).

Despite its name, CPXV is not enzootic in cattle; instead, cattle are incidental hosts of CPXV (Kurth and Nitsche, 2012). Bovine cowpox is extremely rare, with no virologically confirmed cases reported in Great Britain in recent years, and serological surveys show a prevalence of only 0.7% (Baxby, 1977). CPXV has been rarely reported in dogs (Smith et al., 1999; von Bomhard et al., 2011) and horses (Franke et al., 2016), reflecting its wide host range.

2.5. Cowpox virus infection in non-domestic animals

CPXV affects a broad range of non-domestic animals, with significant morbidity and mortality, and new CPXV incidental hosts are still being discovered and reported (Kurth and Nitsche, 2012; Silva et al., 2020).

In zoological settings, cowpox has been reported in multiple families (Table 2.1). Exotic felids are very susceptible to cowpox, particularly cheetah (Marennikova et al., 1977; Baxby et al., 1982; Stagegaard et al., 2017).

CPXV infections have been seen in Asian elephants (*Elaphas maximus*) and African elephants (*Loxodonta africana)* in zoological collections of continental Europe (Table 2.1), but not in the United Kingdom. Nowadays, some zoos vaccinate their elephant herds using the attenuated modified vaccinia virus Ankara (MVA) strain of vaccinia virus, which provides reliable protection against OPV infections; therefore, only sporadic cases still occur in unvaccinated elephants (Kurth and Nitsche, 2012).

The clinical disease seems to vary between species, with two clinical forms being reported (Marennikova et al., 1977): the dermal or cutaneous form, with different degrees of severity; and the pulmonary or systemic form, often with a fatal outcome. Although most cases of cowpox virus in non-domestic animals have proved to be fatal, recovery has often been reported (Baxby and Ghaboosi, 1977; Baxby et al., 1982; Stagegaard et al., 2017; Ashpole et al., 2020).

Table 2.1 - Reports of cowpox virus infection in non-domestic animals* by family, species, geographic origin and year of infection.

Family, Common name	Scientific name	Geographic origin	Year of infection	Reference
Felidae				
Cheetah	*Acinonyx jubatus*	Russia	1973	Marennikova *et al.,* 1977
		Russia	1974	Marennikova *et al.,* 1977
		England	1977	Baxby *et al*., 1982
		Denmark	2010, 2011, 2012, 2014	Stagegaard *et al*., 2017
Lion	*Panthera leo*	Russia	1973	Marennikova *et al.,* 1977
Leopard[a]	*Panthera padus*	Russia	1973	Marennikova *et al.,* 1977
Ocelot	*Leopardus pardalis*	Russia	1973	Marennikova *et al.,* 1977
Puma	*Puma concolor*	Russia	1973	Marennikova *et al.,* 1977
Jaguar	*Panthera onca*	Russia	1973	Marennikova *et al.,* 1977
Leopard cat	*Prionailurus bengalensis*	Russia	1974	Marennikova *et al.,* 1977
Jaguarundi	*Herpailurus yagouaroundi*	Germany	2008	Kurth *et al*., 2009
Elephantidae				
Asian elephant	*Elaphas maximus*	Germany	1960-1990	Pilaski and Jacoby 1993; Baxby and Ghaboosi 1977; Baxby *et al.,* 1979
		Germany	1997	Wisser *et al.,* 2001
		Germany	2007	Kurth *et al.,* 2008
		Czech Republic	1972	Pilaski and Jacoby 1990
		The Netherlands	1973	Pilaski and Jacoby 1991
		Austria	1974	Kubin *et al.,* 1975
		Poland	1977	Pilaski and Jacoby 1992
African elephant	*Loxodonta africana*	Germany	1960-1990	Pilaski and Jacoby 1993
Rhinocerotidae				
Black rhino	*Diceros bicornis*	Germany	1977	Pilaski and Jacoby 1993
		Germany	2004	Eulenberger *et al.,* 2006
White rhino	*Ceratotherium s. simum*	Germany	1977	Pilaski and Jacoby 1993
Camelidae				
Llama	*Lama lama pacos*	Germany	1994	Schüppel *et al.,* 1997
		Italy	2009	Cardeti *et al.,* 2011

Giraffidae				
Okapi	*Okapia johnstoni*	Denmark	1963	Basse *et al.,* 1964
		The Netherlands	1968	Zwart *et al.,* 1971
Ailuridae				
Red panda	*Ailurus fulgens*	Germany	1997	Hentschke *et al.,* 1999
Herpestidae				
Banded mongoose	*Mungus mungo*	Germany	2008	Kurth *et al.,* 2009; Schmiedeknecht *et al.,* 2010
Myrmecophagidae				
Giant anteater	*Myrmecophaga tridactyla*	Russia	1973	Marennikova *et al*., 1977
		England	2014	Ashpole *et al*., 2020
Castoridae				
North American beaver	*Castor canadensis*	Germany	1977	Hentschke *et al*., 1999
Caviidae				
Patagonian cavy	*Dolichotis patagonum*	The Netherlands	2006	Kik *et al*., 2006
Cercopithecidae				
Barbary macaque	*Macaca sylvanus*	The Netherlands	2003	Martina *et al*., 2006
Southern pig-tailed macaque	*Macaca nemestrina*	The Netherlands	2003	Martina *et al*., 2006
Japanese macaque	*Macaca fuscata*	The Netherlands	2003	Martina *et al*., 2006
Nicobar crab-eating macaque	*Macaca fascicularis*	The Netherlands	2003	Martina *et al*., 2006
Rhesus monkey	*Macaca mulatta*	The Netherlands	2003	Martina *et al*., 2006
Callitrichidae				
Common marmoset	*Callithrix jacchus*	Germany	2002	Mätz-Rensing *et al*., 2006
Saddleback tamarin	*Leontocebus fuscicollis*	Germany	2002	Mätz-Rensing *et al*., 2006
Cebidae				
Guianan squirrel monkey	*Saimiri sciureus*	Scotland	2011	Girling *et al*., 2011

*except animals from the superfamily Muroidea.
[a]Black panther.

2.6. Cowpox virus infection in humans

Most human cowpox infections occur via direct contact with an infected cat (Czerny et al., 1991; Baxby et al., 1994; Stewart et al., 2000; Coras et al., 2005; Herder et al., 2011; Haddadeen et al., 2020; Krankowska et al., 2021), and it is estimated that over 50% of human CPXV cases in the United Kingdom are related to exposure to cats (Lawn, 2010). However, confirmed and suggested human CPXV infections from dogs (Pelkonen et al., 2003), cattle (Baxby, 1977), rats (Postma et al., 1991; Stewart et al., 2000; Wolfs et al., 2002; Kurth et al., 2009), elephants (Gehring et al., 1972; Kurth et al., 2008; Hemmer et al., 2010) and cheetahs(Silva et al., 2020) have been reported. Table 2.2 summarises some of the reports of human cowpox by geographic origin, year and likely source of infection.

Similarly to the observed disease in cats (Pastoret et al., 2000), cowpox in humans is most frequently seen during the late summer and autumn, probably reflecting the incidence of feline cases when the number and activity of wild rodents is maximal (Baxby et al., 1994). Cowpox infection in humans is thought to occur via a break in the skin, such as a scratch (Coras et al., 2005), and the hand and face are the most commonly affected sites (Baxby et al., 1994). Although cowpox is generally regarded as a self-limiting cutaneous disease in immunocompetent patients, it may lead to serious complications (Krankowska et al., 2021). Moreover, immunosuppressed patients can develop a widespread and often systemic disease resembling smallpox and often with a lethal outcome (Czerny et al., 1991; Bennett and Baxby, 1996; Vorou et al., 2008; Lawn, 2010; Gazzani et al., 2017; Wendt et al., 2021).

Some authors suggest that the numerous reports of CPXV infection affecting young people in Europe indicate that the lack of smallpox vaccination, which has been abandoned since 1977, may render the population more vulnerable to cowpox virus (Vorou et al., 2008; Żaba et al., 2017), while others believe that the decline in OPV immunity after the cessation of smallpox vaccination is unlikely to affect the incidence of human cowpox (Bennett and Baxby, 1996). These authors believe that although immunisation with smallpox vaccine might prevent the development of severe and systemic cowpox infection, it is unlikely to protect against infection and the development of a primary lesion (Baxby, 1993).

Table 2.2 - A brief summary of some of the reports of human cowpox by geographic origin, year of infection, and likely source of infection.

Geographic origin (Region)	Year of infection	Source of infection	ProMED* Archive Number and/or Reference
Germany	2021	Cat	Wendt *et al*., 2021
Poland	2021	Cat	Krankowska *et al*., 2021
United Kingdom	2021	Cat	Kiernan *et al*., 2021
United Kingdom (Wales)	2018	Cattle, suspected	20180618.5861741
Germany	2017	Unkown	Gronemeyer *et al*., 2017
USA (Georgia)	2011	Laboratory	20110209.0444
France	2011	Rodent (pet rat)	20110629.1982 Elsendoorn 2011
France	2009	Rodent (pet rat)	20090306.0938
France	2009	Rodent (pet rat)	20090226.0809
Germany	2009	Rodent (pet rat)	Schwarzer *et al*., 2013
Germany	2009	Unkown	Strenger *et al*., 2008
Germany	2009	Cat, suspected	Haase *et al*., 2010
Germany	2008	Rodent (pet rat)	20090225.0786 Becker *et al*., 2009
Germany	2007	Cat	Bonnekoh *et al*., 2007
Germany (Northern Germany)	2007	Cat	20070419.1286 Nitsche *et al*., 2007 Nitsche and Pauli 2007
Finland	2003	Dog (suspected)	20070419.1286 Pelkonen *et al*., 2003
The Netherlands	2002	Rodent	20070419.1286 Wolfs *et al*., 2002
Germany	2001	Cat	Coras *et al*., 2005
Ukraine	2001	Cattle	20010508.0888

*Data extracted from the ProMED database.

3. DETECTION OF ORTHOPOXVIRUS ANTIGEN IN ANIMAL TISSUES USING IMMUNOHISTOCHEMISTRY AND TRANSMISSION ELECTRON MICROSCOPY

Immunohistochemistry is a very useful semi-quantitative tool widely used in human and veterinary pathology which uses antibodies to determine distribution and location of cell and protein expression in tissue sections (Duraiyan et al., 2012; Magaki et al., 2019). The method is most commonly performed on FFPE tissues, and the target protein is visualised with brightfield microscopy. Immunohistochemistry involves a series of standardised steps, typically beginning with antigen retrieval. Antigen retrieval processes may involve physical or chemical treatments, with the ultimate goal of unmasking antigens hidden by formalin cross-links or other fixation (Cregger et al., 2006), making them more accessible to antibody binding (Shi et al., 1991).

Primary antibodies can be either monoclonal or polyclonal; monoclonal antibodies target a single epitope and are more specific, while polyclonal antibodies target many different epitopes and are more sensitive (Cartun et al., 2018). When establishing an IHC protocol for the first time, different dilutions of the primary antibody are tested to determine the optimal contrast between positive staining and nonspecific background staining, whilst using the greatest antibody dilution to prevent waste (Lin and Chen, 2014; Taylor, 2014). This may be incorporated with various combinations of dilutions of the secondary antibody in the setting of the particular antigen retrieval method and chromogen to produce optimum staining (Taylor, 2014). The challenges of establishing an IHC protocol include the selection of the primary antibody and the ideal antigen retrieval method and antibody dilution to be used.

The histological observation of A-type eosinophilic intracytoplasmic inclusion bodies (ICIBs) in epithelial cells is extremely suggestive of OPV infections. And although A-type eosinophilic ICIBs are abundant in certain species, making the diagnosis very straightforward, in other species they can be very rare, making the diagnosis more challenging. In such cases, ancillary tests are a very useful tool to reach a diagnosis.

The primary objective of this study was to improve the diagnostic capabilities of the Veterinary Pathology Diagnostic Service of the University of Liverpool by developing an IHC protocol to identify OPV-antigen in FFPE tissues, which could be used as a tool for confirmation in suspected cases of OPV infection. The presence of OPV viral particles in tissue was also assessed by transmission electron microscopy (TEM).

Materials and methods

Sample selection and histopathology: For this study a skin sample from a domestic cat and from a cheetah with OPV type-A eosinophilic ICIBs (Figure 3.1) were used as positive controls, and a skin sample from a healthy cheetah was used as a negative control. Positive and negative controls were run together with the test samples. These skin samples were collected during post-mortem examination (PME) and fixed in 10% neutral buffered formalin for at least 24h. Formalin-fixed samples were processed, sectioned, and stained with haematoxylin and eosin (HE) using standard histology procedures, and slides were examined under a brightfield microscope.

Immunohistochemistry: Two anti-vaccinia virus antibodies targeted to the OPV A27L fusion protein were selected and tested as primary antibodies at different concentrations (Table 3.1): the mouse monoclonal IgG2a anti-vaccinia virus antibody TV46 (atbTV46, Novus Biologicals #NB110-17317) at 1:200, 1:300, 1:500, 1:1000, and 1:2000; and the rabbit polyclonal IgG anti-vaccinia virus antibody A27L (atbA27L, Abcam #ab35219) at 1:100, 1:500, and 1:1000. The IHC was performed using the Dako Autostainer Link 48 (Dako, Denmark), following manufacturer's instructions and reagents provided, unless stated otherwise. Three antigen retrieval methods were tested and included protease (P8038, Sigma), citrate buffer (Low Flex TRS, pH 6.1, K8005) and Tris/EDTA buffer (High Flex TRS, pH9, K8004) (Table 3.1). Before staining, sections were dried, deparaffinized and rehydrated followed by antigen retrieval for 5 minutes. The automated staining procedure consisted of application of EnVision™ FLEX Peroxidase-Blocking Reagent (SM801) for 5 minutes, followed by incubation with the primary antibody for 20 minutes, and then in a peroxidase-labeled polymer (EnVision™ FLEX/HRP, SM802) for 20 minutes, and finally application of the substrate chromogen (EnVision™ FLEX DAB+ Chromogen DM827 and EnVision™ FLEX Substrate Buffer SM802) for 10 minutes. After each step, the sections were rinsed in buffer (EnVision™ FLEX Wash Buffer, K8007). After the final wash step, the slides were counterstained with haematoxylin (EnVision™ FLEX Hematoxylin, K8008, Dako), dehydrated, cleared and mounted with DPX.

Immunolabelled slides were evaluated using brightfield microscopy and a positive immunohistochemical result was determined by the presence of dark brown intracytoplasmic staining on light microscopy. Photomicrographs were acquired with the Leica Application Suite X software using a Leica DMC 4500 digital camera (Leica Microsystems, Switzerland) mounted on a Nikon Eclipse 80i bright field microscope.

Table 3.1 - Primary antibodies, concentrations and antigen retrieval methods tested for the immunohistochemical diagnosis of *Orthopoxvirus* infection.

Antibody		Concentrations	Antigen retrieval methods
atbTV46	mouse monoclonal IgG2a anti-vaccinia virus antibody TV46 (Novus Biologicals #NB110-17317)	1:200 1:300 1:500 1:1000 1:2000	Protease Citrate Tris/EDTA
atbA27L	rabbit polyclonal IgG anti-vaccinia virus antibody A27L (Abcam #ab35219)	1:100 1:500 1:1000	Protease Citrate Tris/EDTA

Transmission electron microscopy: A 20µm section of FFPE skin of the cheetah with OPV type-A eosinophilic ICIBs that was used as a positive control for the IHC was dewaxed in xylene and rehydrated through a descending concentration of ethanol (E047, Taab Laboratories Equipment Ltd., Aldermaston, UK) to distilled water. The section was then fixed in 2.5% buffered glutaraldehyde (G002, Taab), post fixed in 1% osmium tetroxide (O001, Taab), 'en bloc' stained with uranyl acetate (U007, Taab) and dehydrated in an ascending concentration of ethanol and into 100% acetone (A018, Taab). The slide was then immersed in an ascending series of epoxy resin:acetone solutions and finally to 100% epoxy resin (T001, D025, M011, D032, Taab). A beem capsule containing partially polymerised epoxy resin was inverted over the area of interest in the section, and fully polymerised overnight at 60ºC. The slide plus beem capsule was dropped into liquid nitrogen and the beem capsule snapped off, the face of which, contained the area of section of interest. Ultra-thin sections (60-90nm) were cut with a DiATOME diamond knife (AGG3397, Agar Scientific, Essex, UK) on a Reichert-Jung Ultracut ultramicrotome (Leica Microsystems, Milton Keynes, UK), mounted on 200 mesh copper grids (AGG2700C, Agar Scientific), contrast stained with saturated solution uranyl acetate (U007, Taab) in 50% methanol (M023, Taab) followed by 'Reynold's Lead citrate' stain (L037, Taab). The section was analysed and images obtained using a Phillips EM208S transmission electron microscope (Thermo Fisher Scientific, FEI Company, Oregon, USA) at 80kv.

Results

The best contrast between positive staining and nonspecific background staining was obtained using the atbA27L as primary antibody, at a 1:1000 dilution, and protease as AR method. Although the multiple primary antibody concentrations (1:200 to 1:2000) and the three different antigen retrieval methods tested, the

atbTV46 antibody failed to detect A27L protein in FFPE tissues. Positive staining was observed as a brown intracytoplasmic staining, which was restricted to epithelial cells (Figure 3.2) and corresponded to the epithelial cells containing type-A eosinophilic ICIBs observed on HE (Figures 3.1B and 3.2B). No positive IHC staining was observed on the skin sample of the healthy cheetah used as negative control (Figure 3.3).

Despite severe cellular disruption, TEM demonstrated the presence of extracellular barrel-shaped mature virions in the skin sample of the cheetah with OPV type-A eosinophilic ICIBs used as a positive control (Figure 3.4), confirming that the eosinophilic inclusions observed on HE were indeed OPV viral inclusions. The OPV viral particles measured approximately 264 X 163 nm (average measurement of five virions) and were characterised by a dumbbell-shaped core with lateral bodies and an outer lipid membrane bilayer (Figure 3.4).

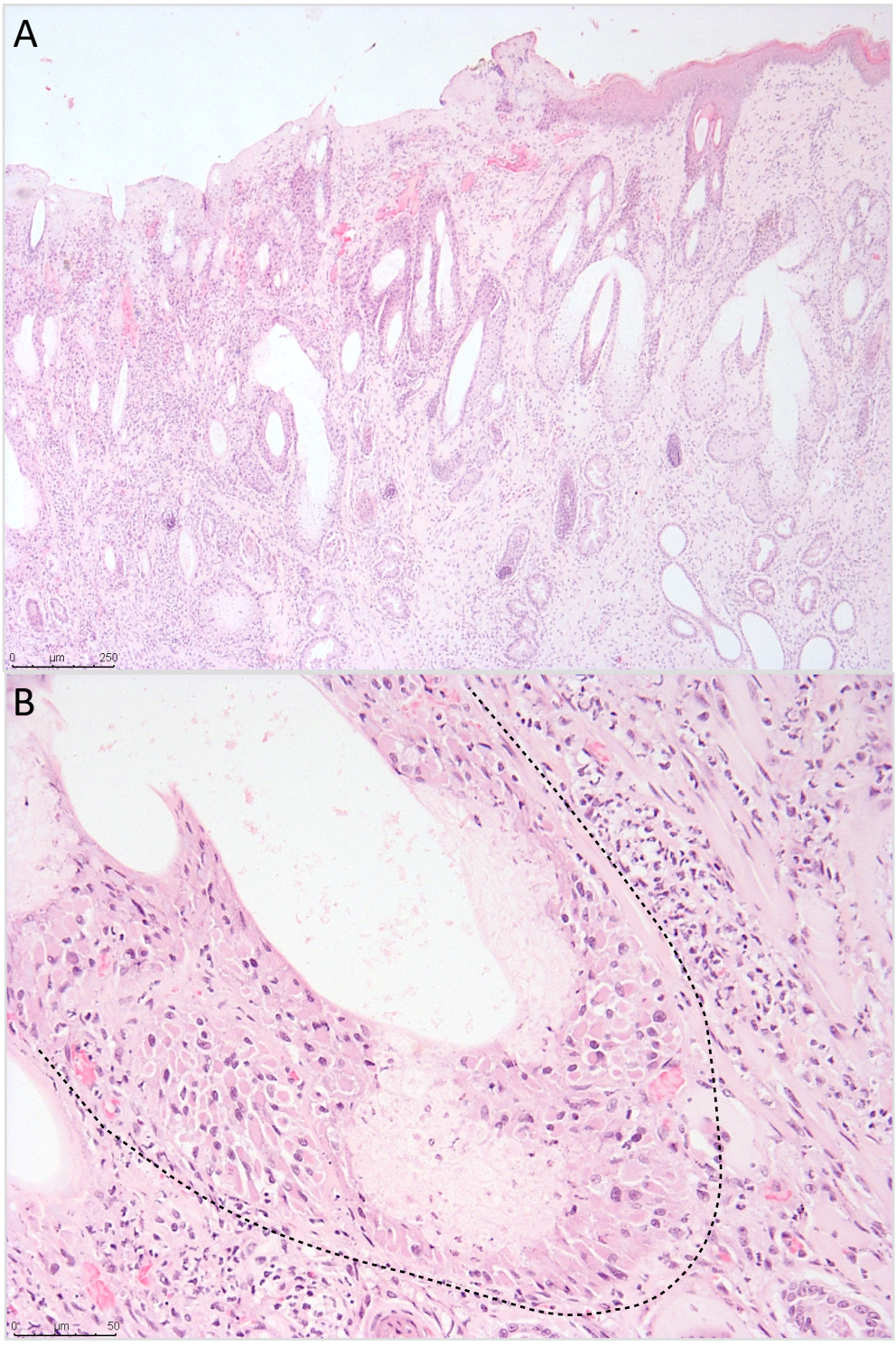

Figure 3.1 - Photomicrograph of the histopathological changes observed on the skin of a cheetah infected with cowpox virus. (A) Delimitation between healthy (right) and affected (left) skin (4X, scale bar 250µm, HE). (B) Multiple characteristic large A-type eosinophilic intracytoplasmic inclusion bodies (star) in the follicular epithelium (hair follicle delimited by dashed line) (20X, scale bar 50µm, HE).

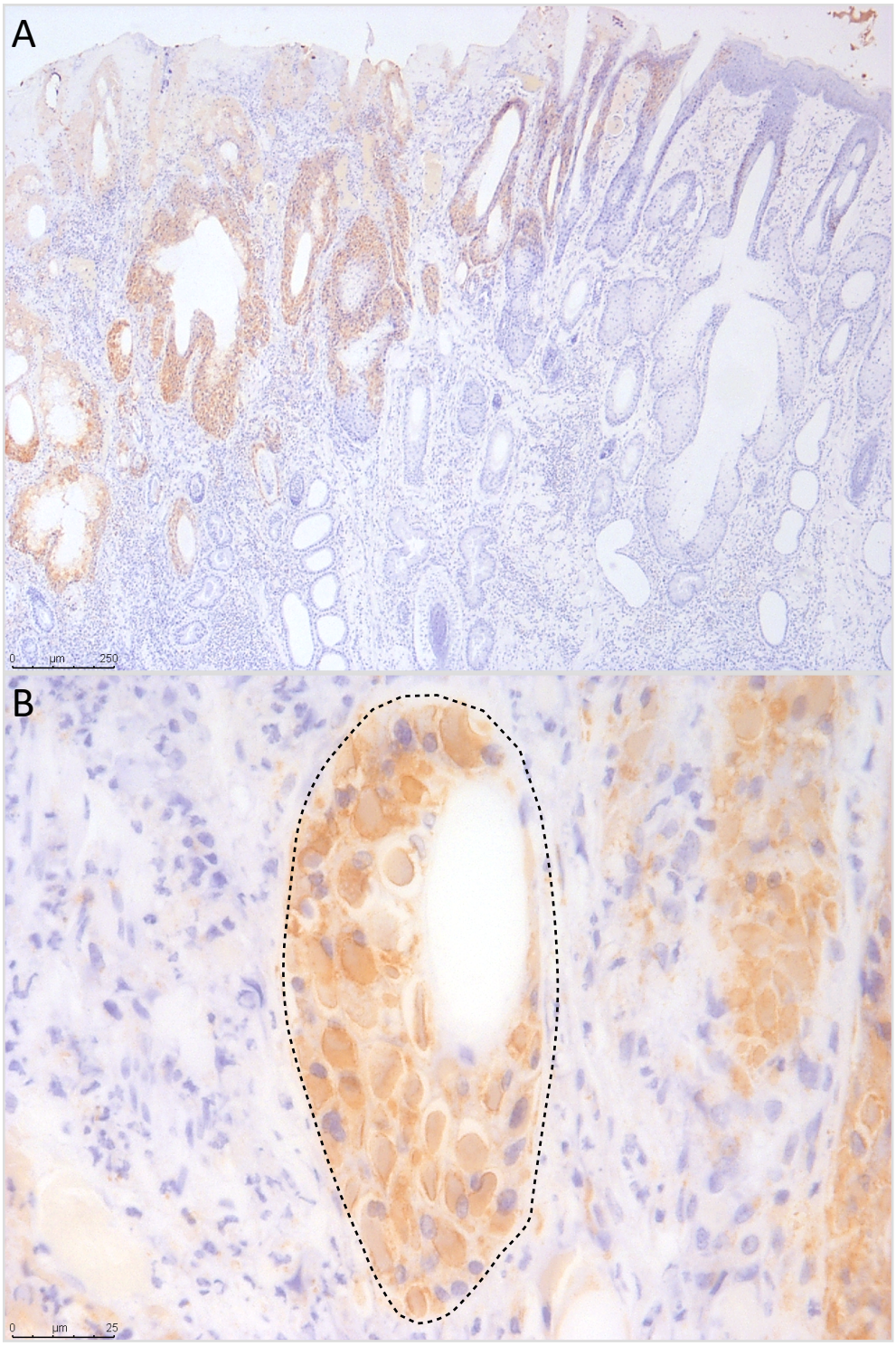

Figure 3.2 - Immunohistochemical staining for OPV A27L fusion protein of the cheetah skin shown in figure 3.1. (A) Delimitation between healthy skin (right), without positive IHC staining, and affected skin (left), with marked positive IHC staining (4X, scale bar 250µm, IHC to OPV A27L fusion protein). (B) Detail of the intracytoplasmic brown staining in follicular epithelial cells (hair follicle delimited by dashed line) (B, 40X, scale bar 25µm, IHC to OPV A27L fusion protein).

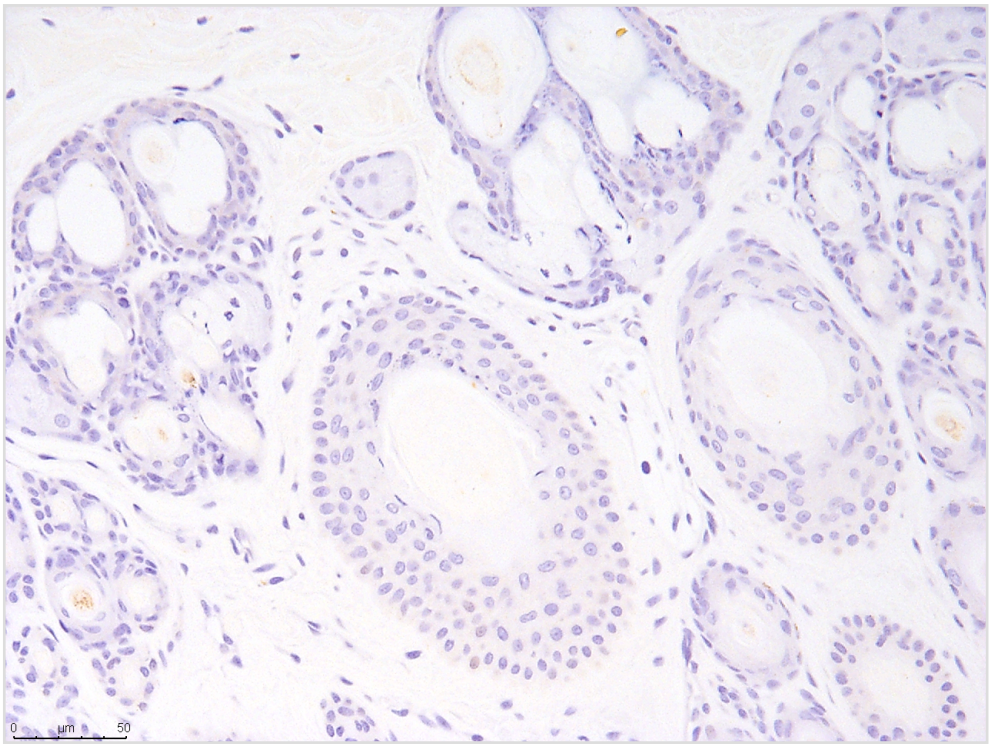

Figure 3.3 - Photomicrograph of the skin of a healthy cheetah used as a negative control (20X, scale bar 50µm, IHC to OPV A27L fusion protein).

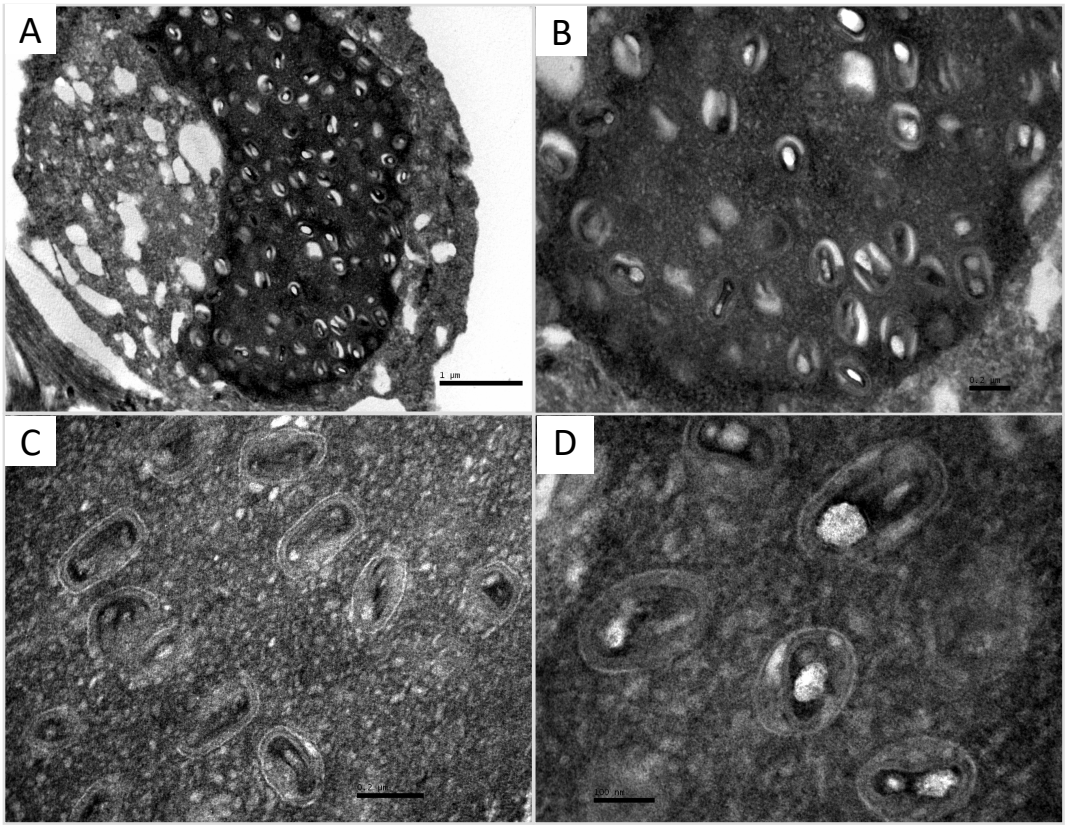

Figure 3.4 - Transmission electron microscopy photomicrograph of the skin of a cheetah with *Orthopoxvirus* infection, showing typical barrel-shaped mature virions a with a dumbbell-shaped core, lateral bodies and an outer lipid membrane bilayer (A, scale bar 1µm; B, scale bar 0.2µm; C, scale bar 0.2µm; D, scale bar 100nm).

Discussion and Conclusions

This study successfully established an IHC protocol for the detection of OPV A27L fusion protein in FFPE tissues of cat and cheetah. At a 1:1000 dilution, and using protease as AR method, the atbA27L proved to be a reliable antibody to detect OPV A27L protein in FFPE tissues. According to the manufacturers, the atbTV46 was optimised for Western blot and ELISA, while the atbA27L was optimised for ELISA and IHC on frozen tissue sections. With the results presented here, we can conclude that the atbA27L is also suitable for IHC of FFPE tissue sections.

Severe disruption of cellular architecture is expected on TEM of FFPE tissues, and is due to a combination of post-mortem autolysis, formalin fixation and paraffin embedding process, making FFPE tissue samples not an ideal candidate for TEM studies. However, despite this limitation, with this technique we successfully detected the presence of OPV viral particles in the skin of an affected cheetah, confirming that the eosinophilic ICIBs observed histologically indeed contained OPV viral particles. Cellular morphology on TEM is best achieved with tissue samples collected immediately after death (or surgical biopsy samples) fixed in glutaraldehyde.

In conclusion, with this newly established IHC protocol we have improved the diagnostic capabilities of the Veterinary Pathology Diagnostic Service of the University of Liverpool to identify OPV antigen in FFPE tissues. This will certainly contribute to the diagnosis of OPV infection, particularly when the number of eosinophilic ICIBs is low or very low. Further studies are needed to evaluate the suitability of this OPV-antigen IHC protocol on FFPE tissues of other domestic and non-domestic animal species.

4. A RETROSPECTIVE EPIDEMIOLOGICAL STUDY OF CUTANEOUS AND SYSTEMIC ORTHOPOXVIRUS INFECTIONS IN ZOO ANIMALS IN THE UNITED KINGDOM[1]

Cowpox is a zoonosis caused by cowpox virus (CPXV), a dsDNA virus that belongs to the genus *Orthopoxvirus* (OPV), family Poxviridae (Moss, 2007). Although it has been extensively studied, the epidemiology and pathobiology of this viral infection varies between regions and is not fully understood. CPXV is distributed throughout Europe (except the island of Ireland) (Chantrey et al., 1999), Russia, and adjacent areas of Northern and Central Asia. An increasing number of cowpox virus infections in humans and animals have been reported in Europe over the past decade (Damon, 2007; Vorou et al., 2008), including in the United Kingdom (Public Health England, 2019), leading to CPXV to be considered an emerging public health threat (Vorou et al., 2008; Żaba et al., 2017).

Using a combination of serology and PCR of blood cell pellets, studies have conclusively demonstrated that the main reservoir of CPXV in Great Britain are bank voles (*Myodes glareolus*), wood mice (*Apodemus sylvaticus*) and short-tailed field voles (*Microtus agrestis*) (Crouch et al., 1995; Chantrey et al., 1999). Whilst the exact species of rodent implicated in the maintenance and transmission cycle of CPXV likely varies according to geographical location (Pastoret et al., 2000), rodents are considered the likely source of infection (Marennikova et al., 1984; Chantrey et al., 1999; Damon, 2007).

CPXV has been associated with diseases in humans (Czerny et al., 1991; Baxby et al., 1994; Kurth et al., 2008; Kurth et al., 2009; Herder et al., 2011), domestic animals, primarily cats (Schaudien et al., 2007; Herder et al., 2011; Jungwirth et al., 2018) but also dogs (Smith et al., 1999; von Bomhard et al., 2011), horses (Franke et al., 2016), and cattle (Baxby, 1977), and a wide range of non-domestic species. Exotic felids appear to be particularly susceptible to CPXV with outbreaks of diseases reportedly affecting captive animals in the United Kingdom, continental Europe and Russia. Most of the CPXV infections documented in non-domestic felids have occurred in cheetahs (*Acinonyx jubatus*) (Marennikova et al., 1977; Baxby et al., 1982; Stagegaard et al., 2017), but there have also been reports in jaguarundi (*Herpailurus yagouaroundi*) (Kurth et al., 2009), lion (*Panthera leo*), jaguar (*Felis onca*), black panther (*Panthera padus*), puma (*Puma concolor*), ocelot (*Leopardus pardalis*) and leopard cat (*Prionailurus bengalensis*) (Marennikova et al., 1977). The disease has also been reported in Asian elephants (*Elaphas maximus*) (Kubin et al., 1975; Baxby and Ghaboosi, 1977; Baxby et al., 1979; Pilaski and Jacoby, 1993; Wisser et al., 2001; Kurth et al., 2008), African elephants

[1] Submission to Emerging Infectious Diseases is pending.

(*Loxodonta africana*) (Pilaski and Jacoby, 1993), llama (*Lama lama pacos*) (Schüppel et al., 1997; Cardeti et al., 2011; von Bomhard et al., 2011), okapi (*Okapia johnstoni*) (Basse et al., 1964; Zwart et al., 1971), red panda (*Ailurus fulgens*) (Hentschke et al., 1999), banded mongoose (*Mungus mungo*) (Kurth et al., 2009; Schmiedeknecht et al., 2010), black rhino (*Diceros bicornis*) (Pilaski and Jacoby, 1993; Eulenberger et al., 2005), white rhino (*Ceratotherium s. simum*) (Pilaski and Jacoby, 1993), giant anteater (*Myrmecophaga tridactyla*) (Marennikova et al., 1977), North American beaver (*Castor canadensis*) (Hentschke et al., 1999), Patagonian cavy (*Dolichotis patagonum*) (Kik et al., 2006), and multiple species of primates including macaques (*Macaca sylvanus, M. nemestrina, M. fuscata, M. fasciularis, M. mulatta*) (Martina et al., 2006), callitrichids (*Callithrix jacchus*, *Leothocebus fuscicollis)* and cebids (*Saimiri sciureus*) (Mätz-Rensing et al., 2006). The clinical disease varies between species and individuals depending on the inoculation dose, with two clinical forms being reported (Marennikova et al., 1977; Bennett et al., 1989): a cutaneous form, where the lesions are restricted to the skin showing different degrees of severity; and a severe systemic form, where viraemic infection occurs and organ systems other than the integumentary system are affected, often with severe morbidity and a fatal outcome. Although many cases of cowpox virus infection in zoo animals have proven to be fatal, recovery has also often been reported (Baxby and Ghaboosi, 1977; Baxby et al., 1982; Stagegaard et al., 2017; Ashpole et al., 2020).

The aim of this retrospective study was to report a series of clinical cases of OPV infection in zoo animals in the United Kingdom, and to describe the epidemiological data, lesion distribution and diagnostic features of each case.

Materials and Methods

Selection of cases and classification of OPV infection: Records of zoo mammal cases submitted to two diagnostic pathology services specialised in non-domestic species in the United Kingdom from January 2004 to April 2021 were reviewed retrospectively. Cases were retrieved from the databases (Microsoft Word and Microsoft Access) of both services using the keywords ‘cowpox’ and ‘orthopoxvirus’, and were considered positive for OPV when at least one of the following four features was observed: (a) the presence of A-type eosinophilic intracytoplasmic inclusion bodies (ICIBs); (b) positivity for OPV-antigen on immunohistochemical staining; (c) the presence of OPV viral particles on transmission electron microscopy; (d) a positive result by PCR for OPV or CPXV DNA. Cases were OPV or CPXV infection was suspected but none of the following four criteria above were met were excluded from the study.

Epidemiological data was compiled and included animal family and species, sex, age (adult being those considered to have reached sexual maturity), month and year of sample submission, season (winter [Dec-Feb], spring [Mar-May], summer [Jun-Aug], autumn [Sep-Nov]), and distribution of gross lesions. The disease was classified either as cutaneous, when gross and histopathological lesions were restricted to the integumentary system, or systemic, when organ systems other than the integumentary system were affected (such as the respiratory or gastrointestinal tarct) with or without the presence of ICIBs. OPV cases where the clinical history only mentioned cutaneous lesions and where only a skin surgical biopsy or a skin swab were submitted for analysis were classified as cutaneous.

A semiquantitative approach was used to quantify the number of ICIBs in the affected tissues. The area on the tissue with the highest number of ICIBs was selected and one 40X field was analysed. The number of ICIBs on the selected 40X field was counted and classified as follows: occasional (+), when 1-5 ICIBs were observed; moderate (++), when 6-30 ICIBs were observed; abundant (+++), when >30 ICIBs were observed.

Histopathological analysis: Tissue samples used for histopathological analysis were either surgical biopsy specimens or tissues collected during post-mortem examination (PME). Tissue samples were fixed in 10% neutral buffered formalin, routinely processed for histopathological evaluation, stained with haematoxylin and eosin (HE), and analysed using a light microscope. Photomicrographs were acquired with the Leica Application Suite X software using a Leica DMC 4500 digital camera (Leica Microsystems, Switzerland) mounted on a Nikon Eclipse 80i bright field microscope.

Immunohistochemistry: Immunohistochemical staining to identify the OPV A27L fusion protein was performed in FFPE as described earlier (Chapter 3), using the Dako Autostainer Link 48 (Dako, Denmark), the rabbit polyclonal IgG anti-vaccinia virus antibody A27L (atbA27L, Abcam #ab35219) at 1:1000 as a primary antibody, and protease (P8038, Sigma) as antigen retrieval method. Immunolabelled slides were evaluated using light microscopy and a positive immunohistochemical result was determined by the presence of dark brown intracytoplasmic staining. Photomicrographs were acquired with the Leica Application Suite X software using a Leica DMC 4500 digital camera (Leica Microsystems, Switzerland) mounted on a Nikon Eclipse 80i bright field microscope.

PCR for Orthopoxvirus and cowpox virus DNA and gene sequencing: Fresh samples from six cases were tested for the presence of OPV DNA using one of two methods. One method, performed at the University of Liverpool, was a nested PCR targeting the OPV thymidine kinase gene as published elsewhere (Chantrey et al., 1999). Briefly, DNA was extracted and purified using QIAamp® DNA Mini Kit

(Quiagen, Hilden, Germany) according to the manufacturer's instructions, and the final DNA elution step was repeated to maximise yield. A tissue sample from a cheetah with known cowpox virus infection (provided by J. Chantrey, University of Liverpool) was used as a positive control. The nested PCR protocol was based on primer homology with the conserved 14kDa orthopoxvirus fusion protein gene (Meyer et al., 1994). The first stage amplification reaction contained 25µl 5x FIREPOL Master Mix RTL with 12.5Mm MgCl2 (04-12-00125, Solis BioDyne, Estonia), 10pmol each of the forward primer FP1 (5'- ATG GAC GGA ACT CTT TTC CC -3') and reverse primer FP2 (5'- TAG CCA GAG ATA TCA TAG CCG C -3') (Eurofins, Germany) and 2µL of DNA template. The reaction mixtures were made up to 50µl with molecular grade water (95284, Sigma Aldrich, United Kingdom) which was also used as negative control. A MJ Research PTC-200 Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used for PCR amplification. Reactions were cycled at 94°C for 6 minutes, followed by 40 cycles at 94°C for 1 minute, annealing at 50°C for 1 minute, and polymerization at 72°C for 1 minute. The final extension cycle was at 72°C for 10 minutes. The first round amplicon size was 292bp. The reamplification stage reaction mixture contained 25µl 5x FIREPOL Master Mix RTL with 12.5Mm MgCl2 (04-12-00125, Solis BioDyne, Estonia); 10pmol each of the forward primer FP3 (5'- CTG AAT TTT TCT CTA CAA AGG CTG CTA A -3') and reverse primer FP4 (5'- TCA GCG TGA TTT TCC AAC CTA AAT AG -3') (Eurofins, Germany) and 1µL of DNA template from the first round. The mixtures were made up to 50µl with molecular grade water (95284, Sigma Aldrich, United Kingdom). The second stage product was 211bp in size. Reactions were cycled as described above. To visualise samples positive for CPXV, 7µl of each PCR product were separated on a 2% agarose (AGR-500, Web Scientific, United Kingdom)/Tris-acetate-EDTA buffer (15558-026, Invitrogen, United Kingdom) electrophoresis gel, preloaded with Web Green Advance (WG-04, Web Scientific, United Kingdom), run for 90 minutes. Digital images of the gel were obtained using the UVIpro gel documentation system (UVItec Ltd., Cambridge, United Kingdom).

The other method was a PCR targeted to the CPXV-specific sequences of the ORF D11L followed by genome sequencing, performed at the Moredun Research Institute (Penicuik, Scotland, United Kingdom), and allowed further characterisation of the OPV. Briefly, DNA was isolated using the Qiagen DNeasy Kit for tissues. Initial amplification was performed using the pan-pox low-GC PCR 5' and 3' primer pair (5'-ACACCAAAAACTCATATAACTTCT and 3'-CCTATTTTACTCCTTAGTAAATGAT) as previously described (Li et al., 2010). As this PCR does not discriminate between the majority of poxvirus genera, a second PCR was subsequently performed with the CPXV_D11L_forward (5'-AAAACTCTCCACTTTCCATCTTCT-3') and CPXV_D11L_reverse

(5'-GCATTCAGATACGGATACTGATTC-3') primers as described elsewhere (Gavrilova et al., 2010; Maksyutov et al., 2015). Whilst the amplification product is 128bp long, and thus able to confirm the target as CPXV, it is not able to discriminate between different strains of CPXV. Therefore, a final PCR was performed with the primer pair 5'-CCCAAGCTTTTATTTTCTAACGAATGTAACGA-3' (Gavrilova et al., 2010) and CPX_D11L_reverse. The amplification products (~600 bp) from these PCRs were cloned into pGemTeasy, sequenced using Sanger sequencing and the results were compared with the GenBank database (http:// www.ncbi.hih.gov).

Transmission electron microscopy (TEM): TEM was used to evaluate the presence of OPV-like viral particles in some of the cases. Briefly, a 20µm FFPE section was dewaxed in xylene and rehydrated through a descending concentration of ethanol (E047, Taab Laboratories Equipment Ltd., Aldermaston, United Kingdom) to distilled water. The section was then fixed in 2.5% buffered glutaraldehyde (G002, Taab), post fixed in 1% osmium tetroxide (O001, Taab), 'en bloc' stained with uranyl acetate (U007, Taab) and dehydrated in an ascending concentration of ethanol and into 100% acetone (A018, Taab). The slide was then immersed in an ascending series of epoxy resin:acetone solutions and finally to 100% epoxy resin (T001, D025, M011, D032, Taab). A beem capsule containing partially polymerised epoxy resin was inverted over the area of interest in the section, and fully polymerised overnight at 60^0C. The slide plus beem capsule was dropped into liquid nitrogen and the beem capsule snapped off, the face of which contained the area of interest. Ultra-thin sections (60-90nm) were cut with a DiATOME diamond knife (AGG3397, Agar Scientific, Essex, United Kingdom) on a Reichert-Jung Ultracut ultramicrotome (Leica Microsystems, Milton Keynes, United Kingdom), mounted on 200 mesh copper grids (AGG2700C, Agar Scientific), contrast stained with saturated uranyl acetate solution (U007, Taab) in 50% methanol (M023, Taab) followed by 'Reynold's Lead citrate' stain (L037, Taab). The section was analysed and images obtained using a Phillips EM208S transmission electron microscope (Thermo Fisher Scientific, FEI Company, Oregon, USA) at 80kv.

Ethical approval: This project was approved by University of Liverpool's School of Veterinary Science Research Ethics Committee (number 12.03.19).

Results

A total of 9879 zoo mammal cases were available on the two databases. OPV infection was diagnosed in 22 mammals (Table 4.2), representing nine species of seven different families (Tables 4.1 and 4.2): 11 cheetahs, two snow leopards (*Panthera uncia*), three Chilean pudus (*Pudu pudu*), one cotton-top tamarin (*Saguinus oedipus*), one Goeldi's monkey (*Callimico goeldii*), one red panda, one

giant anteater, one aardvark (*Orycteropus afer*), and one Malayan tapir (*Tapirus indicus*). Felids were commonly represented, accounting for 59.1% (13/22) of the cases. Adult individuals represented 63.6% (14/22) of the cases, while juveniles represented 36.4% (8/22) of cases. All juvenile animals were felids. The majority of animals were female, representing 59.1% (13/22) of the cases; 36.4% were male (8/22), and 4.5% (1/22) were of unknown sex. Regarding season, 54.5% (12/22) of the cases occurred in the autumn, 18.2% (4/22) occurred in the summer, and 13.6% (3/22) occurring each in spring and winter. As far as sample collection, 77.3% (17/22) were samples obtained on PMEs, while 18.2% (4/22) were biopsy specimens; one sample (4.5%) was a skin swab (Table 4.1).

Macroscopic OPV lesions were primarily observed in the integumentary, gastrointestinal and respiratory tracts, and included necroproliferative dermatitis, glossitis and stomatitis, necrotising pharyngitis and pneumonia (Figures 4.1 to 4.4).

The CPXV infection was classified as systemic in 63.6% (14/22) of the cases and cutaneous in 36.3% (8/22) of the cases (Table 4.2). Histopathological analysis was performed in 21 cases, and 20 of them (with the exception of the Malayan tapir) showed OPV ICIBs in at least one tissue, including skin, subcutis, eyelid, lip, tongue, oral mucosa/pharynx, and lung (Table 4.2).

The number of ICIBs present in the affected tissues varied between species and individuals. Abundant ICIBs were observed in snow leopard (2/2) and Goeldi's monkey (1/1), while cheetah had abundant (6/11) to moderate (5/11) ICIBs (Figure 4.2). Chilean pudu (3/3; Figure 4.2), Cotton top tamarin (1/1), and red panda (1/1) had moderate ICIBs. The giant anteater had occasional ICIBs, while no ICIBs were observed in the Malayan tapir (Table 4.2).

Table 4.1 - Epidemiological data* of cowpox cases in zoo animals submitted to two specialised diagnostic pathology services in the United Kingdom between January 2004 and April 2021.

Species/ID		Sex	Age (year/ month)	Month/year of sample submission	Season	Sample collection
Felidae						
Cheetah	1	F	Juv (1Y)	Sep/2004	Autumn	PME
	2	F	Juv (3M)	Aug/2014	Summer	PME
	3	F	Juv (4M)	Aug/2014	Summer	PME
	4	M	Juv (5M)	Sep/2014	Autumn	PME
	5	M	Ad (2Y)	Mar/2016	Spring	Skin biopsy
	6	F	Juv (4M)	Nov/2011	Autumn	PME
	7	F	Juv (4M)	Nov/2011	Autumn	PME
	8	F	Ad (10Y)	Aug/2017	Summer	PME
	9	F	Ad (5Y3M)	Sep/2017	Autumn	Skin biopsy
	10	M	Ad (8Y)	Sep/2017	Autumn	PME
	11	M	Ad (10Y5M)	Oct/2017	Autumn	Lip biopsy
Snow leopard	1	M	Juv (5M)	Nov/2009	Autumn	PME
	2	F	Juv (5M)	Nov/2009	Autumn	PME
Cervidae						
Chilean pudu	1	F	Ad (3Y4M)	Dec/2010	Winter	PME
	2	F	Ad (6Y)	Dec/2010	Winter	PME
	3	M	Ad	Dec/2010	Winter	PME
Callitrichidae						
Cotton-top tamarin		U	Ad	Sep/2017	Autumn	PME
Goeldi's monkey		F	Ad (1Y)	Oct/2014	Autumn	PME
Ailuridae						
Red panda		M	Ad (10Y)	Oct/2009	Autumn	PME
Myrmecophagidae						
Giant anteater		M	Ad (7Y4M)	Jul/2016	Summer	PME
Orycteropodidae						
Aardvark		F	Ad (7Y10M)	Mar/2021	Spring	Skin swab
Tapiridae						
Malayan tapir		F	Ad	Mar/2011	Spring	Skin biopsy

*F, female. M, male. U, unknown. J, juvenile. Ad, adult. Y, year. M, month. PME, post-mortem examination.

Table 4.2 - Classification of *Orthopoxvirus* (OPV) infection (cutaneous vs. systemic) and diagnostic features in zoo animals submitted to two diagnostic pathology services in the United Kingdom between January 2004 and April 2021.*

Family, Species, ID		Classification of OPV infection (tissues with ICIBs)	Amount of ICIBs	Presence of OPV-antigen, IHC (tissue)	Presence of OPV virions, TEM (sample)	Presence of OPV DNA, PCR (sample)
Felidae						
Cheetah	1	Systemic (skin, lung)	+++	Positive (skin)	—	—
	2	Cutaneous (skin)	+++	—	—	—
	3	Systemic (skin, lung)	+++	—	—	—
	4	Systemic (skin, lung)	+++	—	—	—
	5	Cutaneous (lip)	++	—	—	—
	6	Systemic (skin, tongue, lung)	++	Positive (skin)	Positive (skin)	—
	7	Systemic (skin, tongue, lung)	++	—	—	—
	8	Systemic (skin, subcutis)	++	—	—	—
	9	Systemic (skin, oral mucosa)	+++	—	—	—
	10	Cutaneous (skin, subcutis)	++	—	—	—
	11	Cutaneous (lip)	+++	—	—	—
Snow leopard	1	Systemic (skin, tongue, lung)	+++	Positive (skin, tongue, lung)	—	—
	2	Systemic (skin, eyelid, tongue, lung)	+++	—	—	—
Cervidae						
Chilean pudu	1	Systemic (tongue, oropharynx)	++	—	—	Positive† (sample not specified)
	2	Cutaneous (skin [perivulvar])	++	—	—	Positive† (sample not specified)
	3	Systemic (skin [scrotal], pharynx)	++	Positive (skin [scrotal], pharynx)	—	—
Callitrichidae						
Cotton-top tamarin		Systemic (tongue)	++	Positive (tongue)	—	—
Goeldi's monkey		Cutaneous (skin)	+++	Positive (skin)	—	Positive (oropharyngeal swab)
Ailuridae						

Red panda	Systemic (oral mucosa, tongue)	++	Positive (oral mucosa, tongue)	—	—
Myrmecophagidae					
Giant anteater	Systemic (skin, tongue, pharynx)	+	Positive (skin, pharynx)	—	Positive (skin)
Orycteropodidae					
Aardvark	Cutaneous	—	—	—	Positive (skin swab)
Tapiridae					
Malayan tapir	Cutaneous	Absent	Negative (skin)	—	Positive† (skin)

*ICIB, A-type intracytoplasmic viral inclusion body. IHC, immunohistochemistry. TEM, transmission electron microscopy. PCR, polymerase chain reaction.
†Cases where the OPV was sequenced and identified as cowpox virus (99.8% identical to the UK CPXV reference strain Brighton Red). +, occasional. ++, moderate. +++, abundant. —, not performed.

IHC for OPV A27L fusion protein was performed in nine cases representing eight species: two cheetahs (cheetah 1 and 6), one snow leopards (snow leopard 1), one Chilean pudu (Chilean pudu 3), one Cotton-top tamarin, one Goeldi's monkey, one red panda, one giant anteater, and one Malayan tapir (Table 4.2). Positivity for OPV antigen was observed in eight of these cases, except for the Malayan tapir; this animal did not have ICIBs on histopathological examination of the skin, but was positive for the presence of CPXV DNA (Table 4.2).

TEM was performed in one cheetah (cheetah 6) and viral particles compatible with OPV were observed (Figure 4.8). The particles showed typical barrel-shaped mature virions, with a dumbbell-shaped core, lateral bodies and an outer lipid membrane bilayer.

Six samples were tested for the presence of OPV DNA (Goeldi's monkey, giant anteater, aardvark, Chilean pudus 1 and 2, and Malayan tapir), and all yielded a positive result. CPXV was confirmed in three of these cases, Chilean pudus 1 and 2, and Malayan tapir (Table 4.2). The sequence obtained from the 013 gene of the Chilean pudus 1 and 2, and the Malayan tapir was almost identical (99.8%, only one base change in 595 bases) to that of the CPXV reference strain Brighton Red, which circulates in Great Britain. In contrast, the isolate was only 98.5% (586/595) identical to strain Germany 91-3 and 96% (575/601: the larger number on the denominator is due to gaps needing to be introduced into the sequence in order to obtain the optimal alignment) identical to the Russian strain GRI-90.

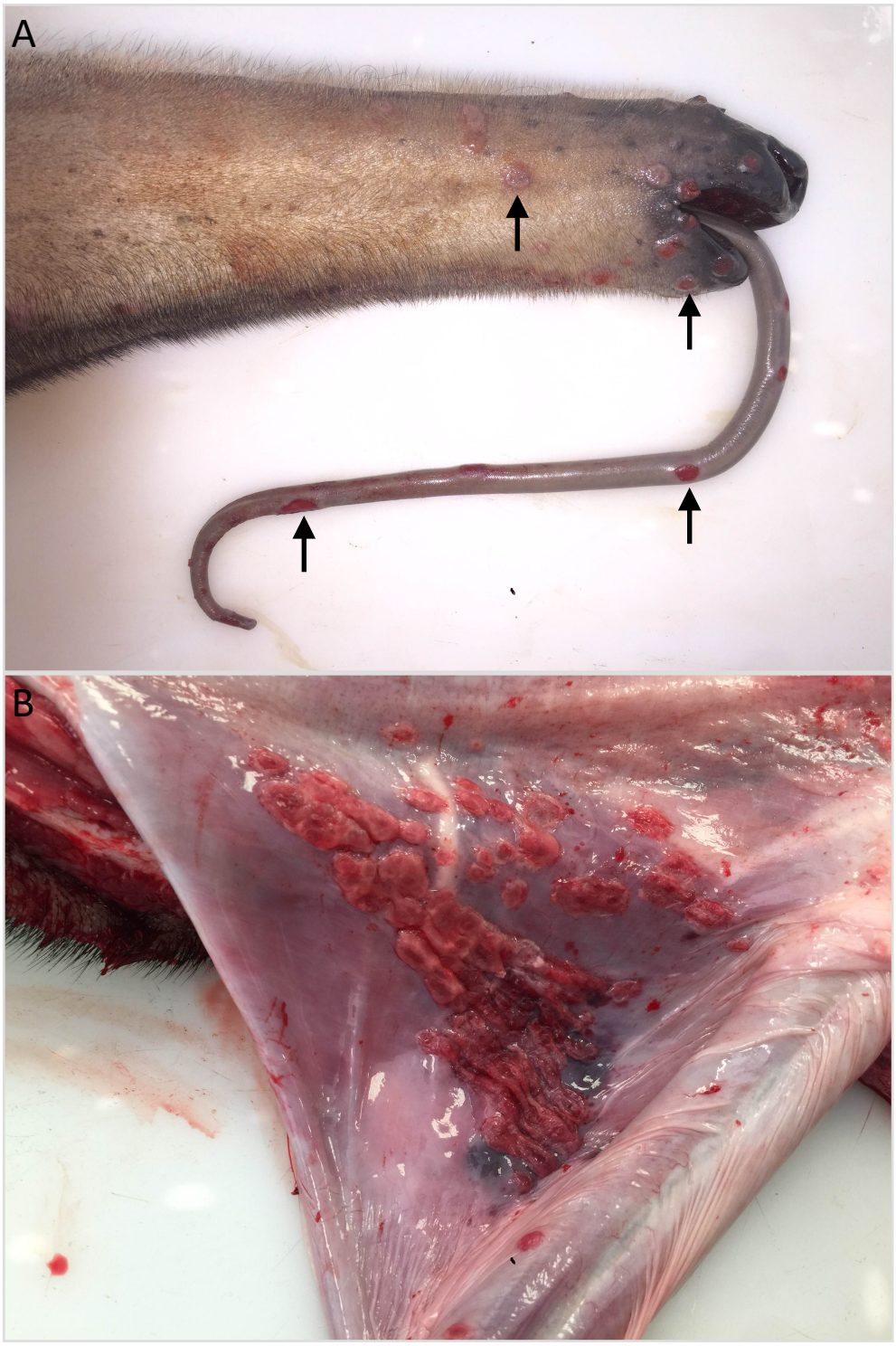

Figure 4.1 - Macroscopic lesions of OPV in a giant anteater. (A) Multifocal necroproliferative lesions in the head, muzzle and tongue. (B) Same giant anteater as image A, showing multifocal to coalescing areas of necroproliferative pharyngitis.

Figure 4.2 - Macroscopic lesions of OPV in a cheetah. (A) Multifocal necroproliferative and ulcerative dermatitis. (B) Skin of the cheetah shown in image A, showing a focal raised area of necroproliferative dermatitis, with a central area of depression.

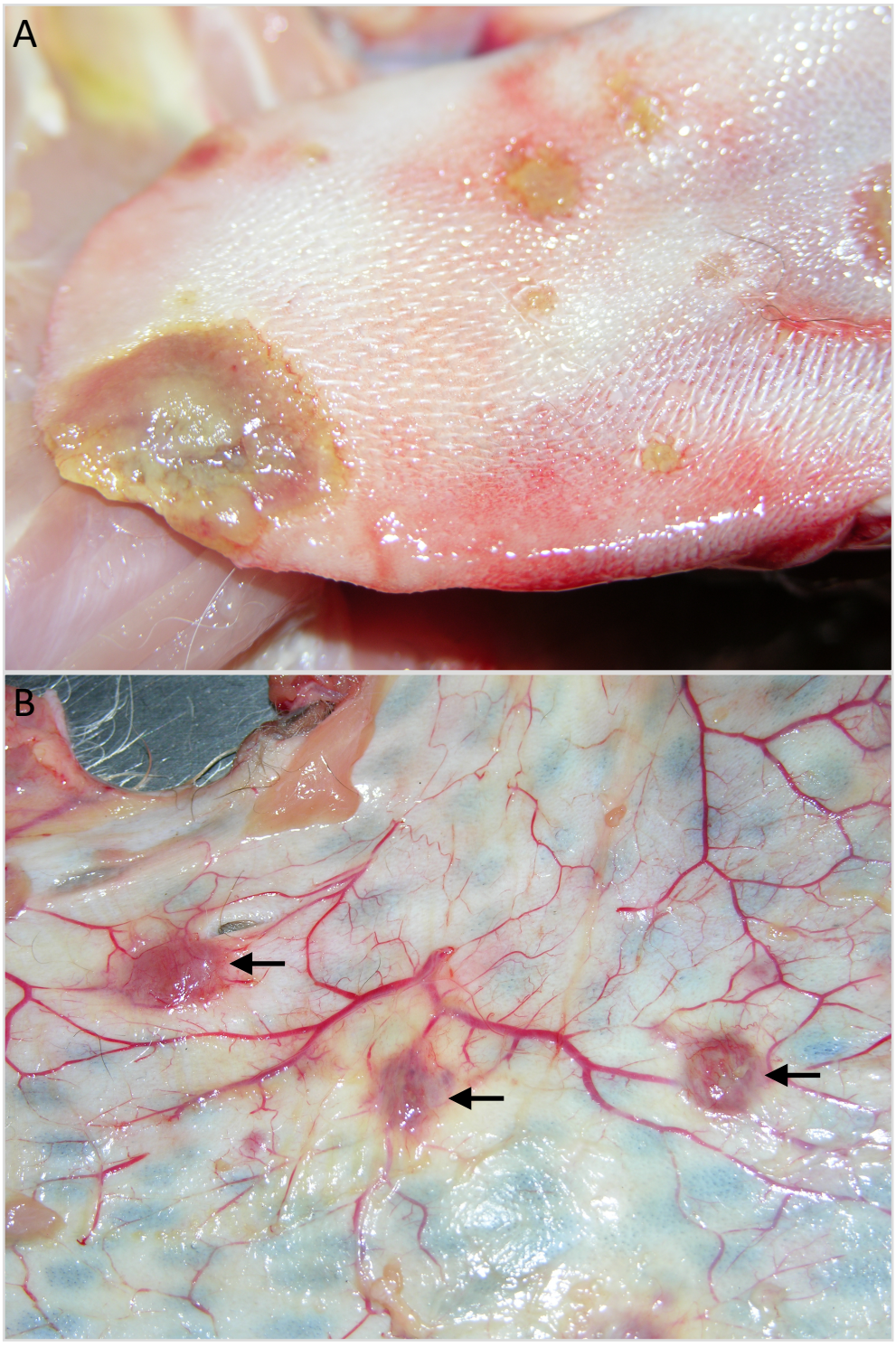

Figure 4.3 - Macroscopic lesions of OPV in a cheetah, same animal shown in Figure 4.2. (A) Tongue of a cheetah, with multifocal areas of necroulcerative glossitis. (B) Skin of a cheetah, with multifocal necroproliferative lesions on the subcutis, targeting blood vessels.

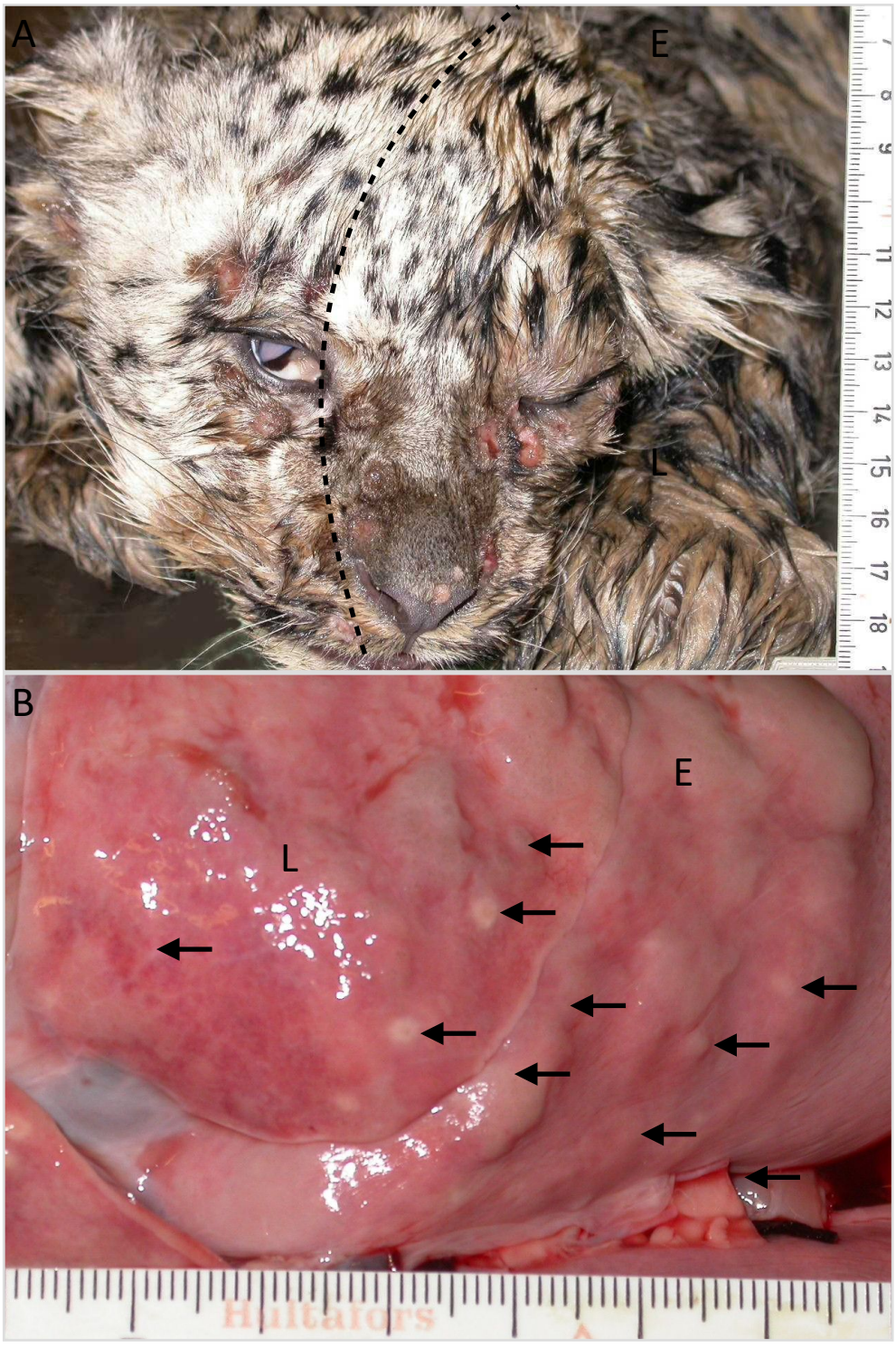

Figure 4.4 - Macroscopic lesions of OPV in a snow leopard.(A) Multifocal to coalescing necroproliferative and ulcerative dermatitis. (B) Lung of the same snow leopard as in image A, with multifocal necrotising pneumonia.

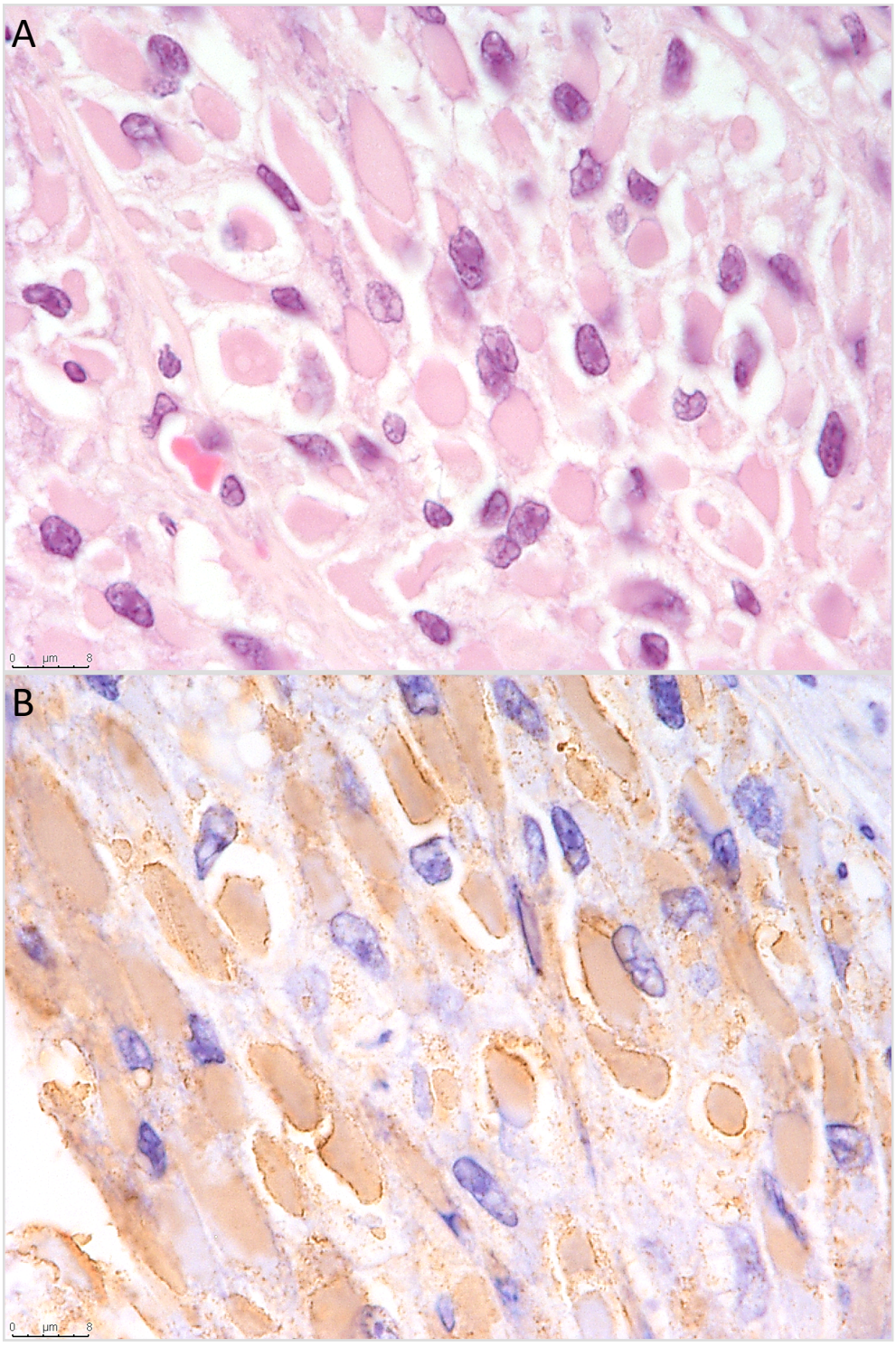

Figure 4.5 - Photomicrograph of the skin of a cheetah infected with CPXV. (A) Characteristic large A-type intracytoplasmic inclusion bodies in epithelial cells (100X, scale bar 8µm, HE). (B) Immunohistochemical analysis of the skin of the cheetah shown in A, with intracytoplasmic brown staining (100X, scale bar 8µm, IHC to OPV A27L fusion protein).

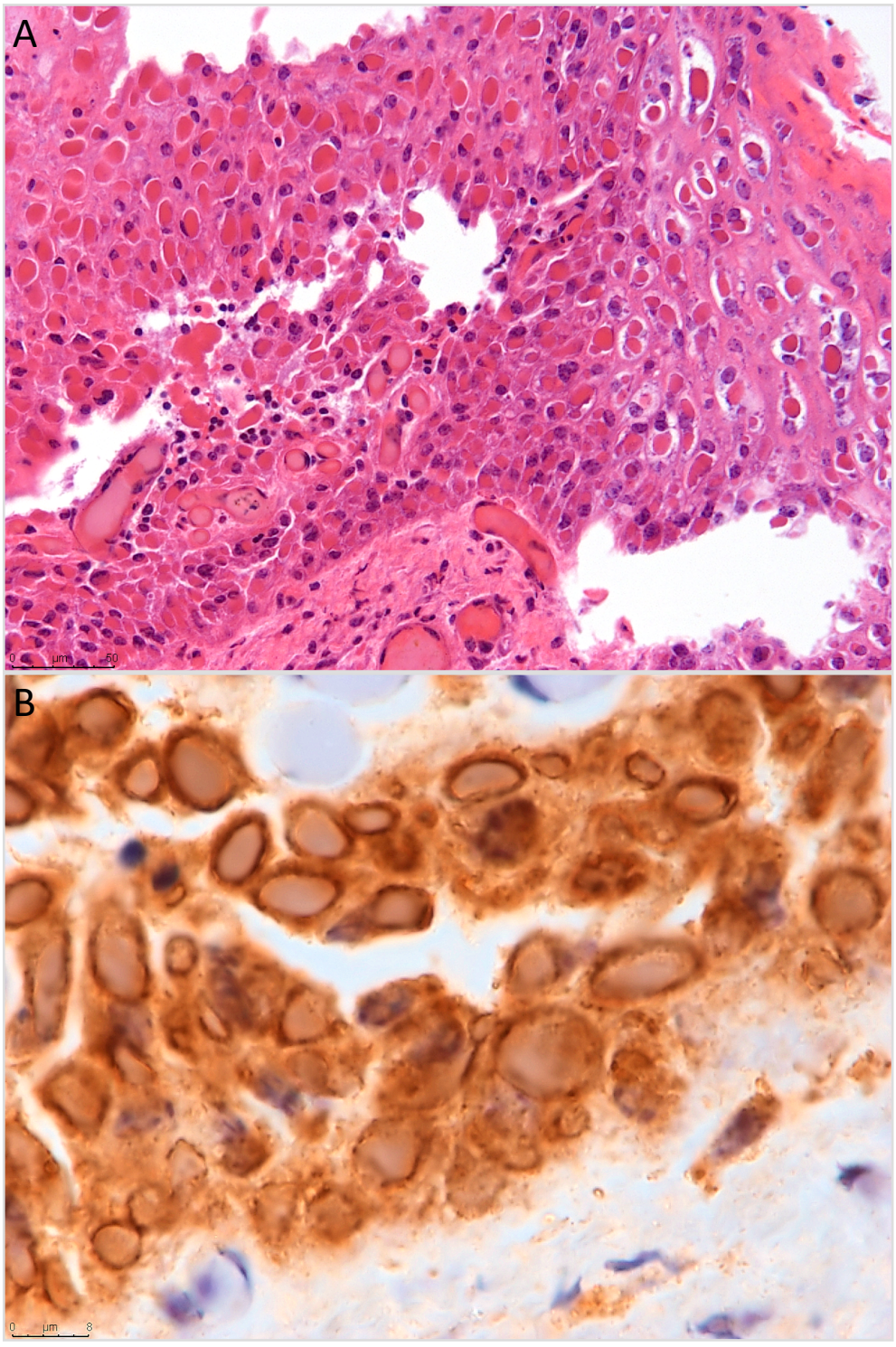

Figure 4.6 - Photomicrograph of the mucous membrane of a Chilean pudu infected with CPXV. (A) Characteristic large A-type intracytoplasmic inclusion bodies in epithelial cells (20X, scale bar 50µm, HE). (B) Immunohistochemical analysis of the mucous membrane of the Chilean pudu shown in A, with intracytoplasmic brown staining (100X, scale bar 8µm, IHC to OPV A27L fusion protein).

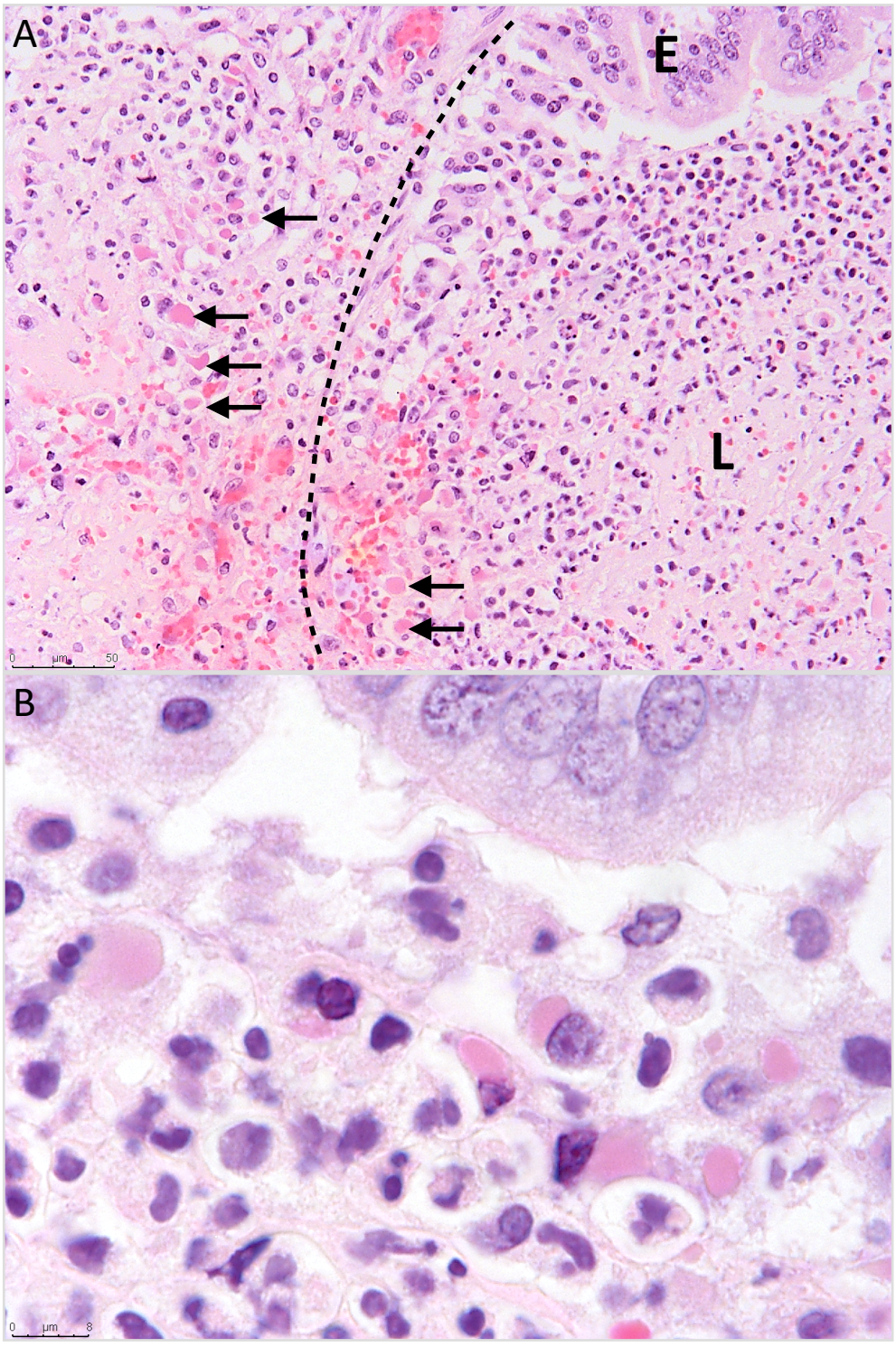

Figure 4.7 - Photomicrograph of the lung of a cheetah infected with CPXV, with characteristic A-type intracytoplasmic inclusion bodies (arrows) in sloughed bronchiolar epithelial cells (A, 20X, scale bar 50µm, HE; B, 100X, scale bar 8µm, HE). Dashed line in A delimits a bronchiole. E: bronchiolar epithelium; L: bronchiolar lumen.

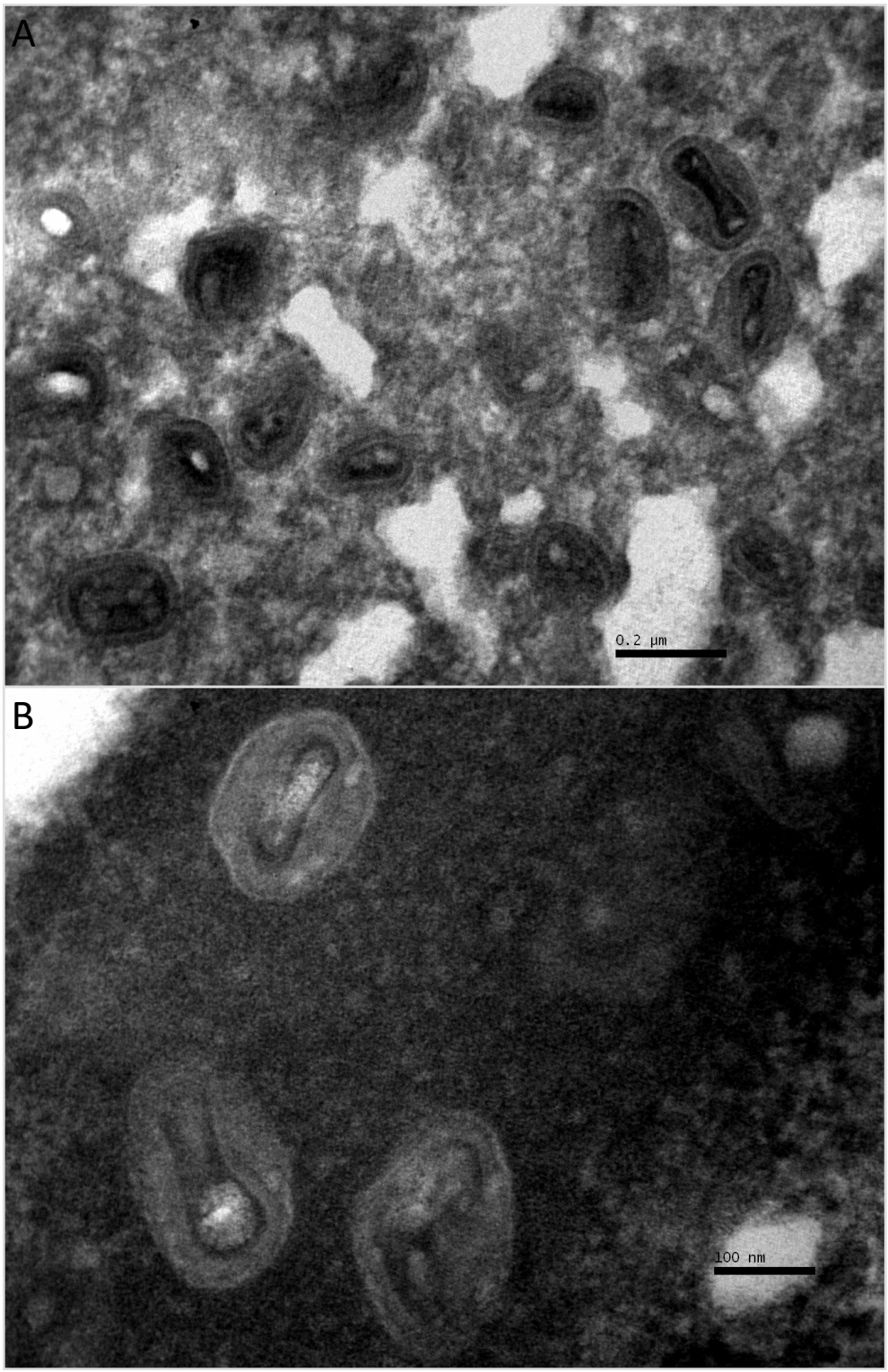

Figure 4.8 - Transmission electron microscopy photomicrograph of the skin of a cheetah with *Orthopoxvirus* infection, showing typical barrel-shaped mature virions with a dumbbell-shaped core, lateral bodies and an outer lipid membrane bilayer.(A, scale bar 0.2µm; B, scale bar 100nm).

Discussion

This retrospective study described for the first time clinical OPV infection in two snow leopards, three Chilean pudus, one aardvark, and one Malayan tapir, suggesting that OPV has an even wider range of susceptible zoo animal species than those previously reported.

Cheetahs represented 50% (11/22) of the total number of cases, reinforcing the theory that most of the OPV infections among non-domestic felids occur in cheetah (Marennikova et al., 1977; Baxby et al., 1982; Stagegaard et al., 2017). Over half of the OPV cases (54.5%, 12/22) were submitted in the autumn (Sep-Nov), which corresponds to the cowpox seasonality previously reported in captive cheetahs (Stagegaard et al., 2017), cats and humans (Baxby et al., 1994), and reflects the population and infection dynamics in wild rodents in the United Kingdom (Chantrey et al., 1999; Pastoret et al., 2000). However, as previously reported (Pfeffer et al., 2002; Godfrey et al., 2004), cases were observed throughout the year, making seasonality an unreliable factor for diagnosing OPV infections. All OPV cases in juvenile animals were in felids, which could be explained by their hunting nature.

This study does not represent the actual number of affected animals in each outbreak, as samples from affected conspecifics are not necessary submitted for diagnostic confirmation. Moreover, as this study is based on samples submitted to diagnostic pathology services, it is biased towards fatal cases.

Cutaneous OPV infections were restricted to the skin or, occasionally, the lip. On the other hand, in systemic infections, OPV ICIBs were observed in the integumentary, digestive and respiratory systems, indicating that OPV is capable of systemic spread and viral replication. Interestingly, cutaneous CPXV lesions on the external genitalia were observed at the perivulvar skin (Chilean pudu 2) and the scrotal skin (Chilean pudu 3) of two out of the three Chilean pudus.

Although the presence of ICIBs on histological examination is highly characteristic of OPV infection, the number of ICIBs varies between species and individuals (Table 4.2) and might, occasionally, be absent (Pfeffer et al., 2002). This could perhaps be associated with the progression of the infection (e.g. higher number of ICIBs might be observed on the acute phase of the infection vs subacute or chronic stages), or with the severity of the lesion (e.g. higher number of ICIBs in more severe lesions vs in lesions of mild severity). Similar comparison studies with large number of individuals per species are encouraged to determine whether these differences are species specific and, if so, their clinical relevance.

In the present study, IHC proved to be a very useful tool to detect OPV-antigen in tissues and, therefore, assist on the diagnosis of OPV infection. As observed in the Malayan tapir, in cases where OPV ICIBs are absent or only

occasionally observed histologically, molecular techniques to detect OPV DNA or, ideally, CPXV DNA are highly encouraged.

Severe disruption of cellular architecture is expected on TEM of FFPE tissues, and is due to a combination of post-mortem autolysis, formalin fixation and the paraffin embedding process, making FFPE tissue samples not ideal for TEM studies. However, despite this limitation, OPV viral particles were successfully detected in the skin of an affected cheetah using this technique, confirming that the eosinophilic intracytoplasmic inclusion bodies observed histologically were indeed OPV viral particles. Cellular morphology on TEM is best achieved with tissue samples collected immediately after death (or surgical biopsy samples) fixed in glutaraldehyde.

Although molecular characterisation of the OPV as CPXV was only performed in three cases (Chilean pudus 1 and 2, and Malayan tapir, from the same zoological collection), the sequence obtained was almost identical (99.8%) to that of the CPXV reference strain Brighton Red. This result suggest that the CPXV responsible for these three cases is very likely to be an enzootic CPXV strain known to be circulating in the United Kingdom.

This retrospective study contributes to the understanding of the epidemiology and species susceptibility of this condition. Moreover, it describes a reliable IHC protocol for the confirmation of orthopoxvirus infection in FFPE tissues that can be implemented in routine diagnostic laboratories.

5. DISEASE SURVEILLANCE IN WILD SMALL MAMMALS AT CHESTER ZOO, WITH EMPHASIS ON COWPOX AND TOXOPLASMOSIS

Introduction

Wild rodent populations, such as brown rats and house mice (*Mus musculus*), are attracted to zoos throughout the year, but particularly during the winter months due to the wide availability of shelter, heated enclosures, and access to food sources (Stidworthy, 2010). Rodents act as the reservoir of a wide range of pathogens that cause diseases in both zoo animals and humans, including CPXV and toxoplasmosis (Stidworthy, 2010).

Toxoplasmosis is a disease caused by the coccidian parasite *Toxoplasma gondii*. The life-cycle of *T. gondii* is complex and involves Felids (domestic cats and their relatives) as the definitive hosts, and rodents as the primary intermediate hosts, However, *T. gondii* is capable of infecting multiple other warm-blooded animal species, including humans, that can also act as intermediate hosts. While *T. gondii* infection is usually asymptomatic in both the definitive and the intermediate hosts, it can lead to acute and severe morbidity and mortality in very susceptible species, such as New World monkeys, Australian marsupials, Pallas's cats, lemurs, canaries and finches (Dubey and Dubey; Dubey, 2010; Guthrie et al., 2017; Marková et al., 2019; Paula et al., 2020). It is a significant protozoan pathogen of zoo animals, causing abortions and polysystemic infections including encephalitis, pneumonia, hepatitis and splenitis (Stidworthy, 2010). Although most cases are associated with exposure to sporulated oocysts present in cat faeces, some cases (e.g. felids and non-human primates) result from ingestion of rodents carrying tissue cysts (Stidworthy, 2010).

Over the last decade, Chester Zoo (Upton-by-Chester, Cheshire, England, 53°13′36″N 2°53′3″W) has suffered several outbreaks of infectious diseases transmitted by wild rodents, including cowpox in cheetahs and giant anteaters (Ashpole et al., 2020), and toxoplasmosis in Pallas's cats (Costa et al., 2020) and meerkats (Julian Chantrey, personal communication). However, the exact epidemiology of these outbreaks is still unknown. The aim of this surveillance study was to assess the prevalence of diseases in wild small mammals at Chester Zoo, with emphasis on CPXV and *T. gondii*, in order to provide a better understanding of the epidemiology of these diseases in this zoological collection.

Materials and Methods

Small mammal trapping: Wild small mammals trapped at Chester Zoo as part of their pest management programme were used for this study. The target trapping periods of the project were autumn 2019 (Sep-Nov) and late winter 2020

(Feb), four days a week, for two weeks during each target month. Lethal snap traps were placed in enclosures where cases of CPXV and toxoplasmosis were known to have occurred in the past, or where species very susceptible to CPXV were housed. Four target locations were identified: 'jaguars', 'miniature monkeys', 'Pallas's cats', and 'cheetahs'. Trapping was focused on two rodent pest species, brown rats and house mice. However, as pest management activities take place throughout the year and on a wide range of locations, the project included every small mammal species trapped, including accidentally trapped non-pest species, throughout Chester Zoo from February 2019 to February 2020.

Snap traps were set in the late afternoon and were checked and removed the following morning. Carcasses were placed in a zipped plastic bag, labelled with trap location and date of collection. Carcasses were then frozen at -20˚C at the Chester Zoo's Animal Health Centre before being transferred to the Department of Veterinary Anatomy, Physiology and Pathology of the University of Liverpool (Institute of Infection, Veterinary and Ecological Sciences, Leahurst Campus, Neston, England), where they were kept frozen at -20˚C until PME was performed. Appropriate personal protective equipment (disposable gloves and face masks) was used when handling traps or trapped animals.

Post-mortem examination, sample collection and histopathology: Carcasses were defrosted overnight at 4°C and PME was performed using standard protocols. Species, age (juvenile/adult/undetermined), sex (male, female, unknown), body weight, and body condition score (1: emaciated; 2: thin; 3: average; 4: overweight; 5: obese) were recorded. Samples were divided by the season they were collected, as follows: winter (Dec-Feb), spring (Mar-May), summer (Jun-Aug), autumn (Sep-Nov). Samples of small intestine, spleen, liver, kidney and brain were collected in duplicate, and were placed in a 1.5 mL Eppendorf Tube® and frozen at -20˚C, and fixed at 10% neutral-buffered formalin for histopathology. Formalin-fixed samples were processed, sectioned, and stained with haematoxylin and eosin (HE) using standard histology procedures, and slides were examined under a light microscope.

Toxoplasma gondii immunohistochemistry: Immunohistochemistry for *T. gondii* was performed using the Dako Autostainer Link 48 (Dako, Glostrup, Denmark). Before staining, sections were dried, deparaffinized and rehydrated followed by antigen retrieval using EnVision™ FLEX Target Retrieval Solution (TRS) Low pH (Citrate buffer, pH 6.1, Agilent K8005) for 5 minutes. The automated staining procedure consisted of application of EnVision™ FLEX Peroxidase-Blocking Reagent (SM801, Dako) for 5 minutes, followed by incubation in Rabbit anti-human *T. gondii* polyclonal antibody (MBS373041, MyBioSource, USA) (dilution 1:80) for 20 minutes, and then in a peroxidase-labeled polymer EnVision™+ System-HRP Labelled Polymer Anti Rabbit (K4003) for 20 minutes, and finally

application of the substrate chromogen (EnVision™ FLEX DAB+ Chromogen DM827 and EnVision™ FLEX Substrate Buffer SM802, Dako) for 10 minutes. After each step, the sections were rinsed in buffer (EnVision™ FLEX Wash Buffer, K8007, Dako). After the final wash step, the slides were counterstained with haematoxylin (EnVision™ FLEX Hematoxylin, K8008, Dako), dehydrated, cleared and mounted. Microscopic images were acquired with the Leica Application Suite X software using a Leica DMC 4500 digital camera (Leica Microsystems, Switzerland) mounted on a Nikon Eclipse 80i bright field microscope.

Nested PCR for Orthopoxvirus DNA (OPV DNA): DNA was extracted from samples of spleen and/or small intestine and purified using QIAamp® DNA Mini Kit (Quiagen, Hilden Germany) according to the manufacturer's instructions for tissue DNA extraction. The final DNA elution step was repeated to maximise yield. A tissue sample from a cheetah with known cowpox infection (provided by J. Chantrey, University of Liverpool) was used as a positive control.

The nested PCR protocol was based on primer homology with the conserved 14kDa orthopoxvirus fusion protein gene (Meyer et al., 1994). The first stage amplification reaction contained 25µl 5x FIREPOL Master Mix RTL with 12.5Mm $MgCl_2$ (04-12-00125, Solis BioDyne, Estonia), 10pmol each of the forward primer FP1 (5'- ATG GAC GGA ACT CTT TTC CC -3') and reverse primer FP2 (5'- TAG CCA GAG ATA TCA TAG CCG C -3') (Eurofins, Germany) and 2µL of DNA template. The reaction mixtures were made up to 50µl with molecular grade water (95284, Sigma Aldrich, UK) which was also used as negative control. A MJ Research PTC-200 Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used for PCR amplification. Reactions were cycled at 94°C for 6 minutes, followed by 40 cycles at 94°C for 1 minute, annealing at 50°C for 1 minute, and polymerization at 72°C for 1 minute. The final extension cycle was at 72°C for 10 minutes. The first round amplicon size was 292bp. The reamplification stage reaction mixture contained 25µl 5x FIREPOL Master Mix RTL with 12.5Mm $MgCl_2$ (04-12-00125, Solis BioDyne, Estonia); 10pmol each of the forward primer FP3 (5'- CTG AAT TTT TCT CTA CAA AGG CTG CTA A -3') and reverse primer FP4 (5'- TCA GCG TGA TTT TCC AAC CTA AAT AG -3') (Eurofins, Germany) and 1µL of DNA template from the first round. The mixtures were made up to 50µl with molecular grade water (95284, Sigma Aldrich, UK). The second stage product was 211bp in size. Reactions were cycled as described above. To visualise samples positive for CPXV, 7µl of each PCR product were separated on a 2% agarose (AGR-500, Web Scientific UK)/ Tris-acetate-EDTA buffer (15558-026, Invitrogen, UK) electrophoresis gel, preloaded with Web Green Advance (WG-04, Web Scientific, UK), run for 90 minutes. The PCR reaction was performed in single reaction tubes and repeated

twice if the first reaction yield a positive result. Digital images of the gel were obtained using the UVIpro gel documentation system (UVItec Ltd., Cambridge, UK).

Ethical approval: This project was approved by the School of Veterinary Science Research Ethics Committee (number 12.03.19) of the University of Liverpool, and by the Animal Health Welfare and Husbandry Committee and the Research Department of Chester Zoo.

Statistical analysis: Descriptive statistical analysis was performed using Minitab® 19.2020.1.

Results

A total of 178 wild small animals of seven different species were trapped, and included the two pest species, brown rat (n=66) and house mouse (n=12), and five species accidentally trapped, wood mouse (n=57), bank vole (n=25), field vole (n=13), harvest mouse (*Microtus minutus*, n=2), and common shrew (*Sorax araneus*, n=3). Post-mortem examination was performed on 118 animals, and included all seven species. The state of post-mortem autolysis of the cadavers varied from minimal to severe. Cutaneous lesions compatible with OPV infection were absent.

As some of the samples were too autolysed for useful histopathological examination and not every sample was collected from every animal, a total of 65 samples of small intestine, 109 samples of brain, 95 samples of kidney, 97 samples of liver, and 83 samples of spleen were subjected to histopathological analysis. Histopathological findings on each species are summarised on Table 5.1. No intracytoplasmic inclusion body compatible with OPV were observed in the small intestinal epithelial cells of the animals examined.

Bradyzoite-containing tissue cysts were observed in the brain of three adult males and one adult female bank voles, trapped at the 'Pallas's cat' enclosure (Figure 5.5). These animals were trapped in the autumn 2019 (Table 5.2), and the tissue cysts were not associated with inflammatory response. Three of them showed positive immunolabeling for *T. gondii* antigen using IHC (Figure 5.5); the fourth case did not have tissue cysts on the section used for *T. gondii* IHC. Although the overall prevalence of toxoplasmosis amongst all brain samples tested was relatively low, 3.7% (4/109), the prevalence in bank voles was relatively high at 16.7% (4/24) (Tables 5.1 and 5.2, Figure 5.5).

Table 5.1 - Histopathological findings in the small intestine, brain, kidney, liver and spleen of seven species of wild small mammals trapped at Chester Zoo, from February 2019 to February 2020.

***Tissue*, histopathological findings**	**Species**							***Total*, n (prevalence)**	**95% CI (%)**
	***Number of samples tested*, number of samples showing lesion**								
	Brown rat	House mouse	Wood mouse	Bank vole	Field vole	Harvest mouse	Common shrew		
Small Intestine	*2*	*6*	*29*	*15*	*11*	*1*	*1*	*65*	
Apicomplexan	0	0	1	1	0	1	0	3 (4.6%)	0.96-12.89
Capillariasis	0	0	2	2	0	0	0	4 (6.2%)	1.7-15.01
Nematodiasis	0	4	16	1	0	0	0	21 (32.3%)	21.23-45.05
Cestodiasis	0	0	0	1	0	0	1	2 (3.1%)	0.38-10.68
Brain	*4*	*10*	*54*	*24*	*12*	*2*	*3*	*109*	
Bradyzoite-containing tissue cysts	0	0	0	4	0	0	0	4 (3.7%)	1.01-9.13
Non-suppurative encephalitis	0	0	1	2	1	0	0	4 (3.7%)	1.01-9.13
Kidney	*4*	*10*	*42*	*23*	*12*	*1*	*3*	*95*	
Tubulointerstitial nephritis	0	0	0	0	1	0	0	1 (1.1%)	0.03-5.72
Pyelonephritis	0	0	1	0	0	0	1	2 (2.1%)	0.25-7.39
Renal coccidiosis	0	1	0	0	0	0	0	1 (1.1%)	0.03-5.72
Intraendothelial meronts	0	0	0	0	0	0	1	1 (1.1%)	0.03-5.72
Liver	*4*	*8*	*47*	*24*	*11*	*1*	*2*	*97*	
Lymphoplasmacytic periportal hepatitis	0	0	7	12	10	0	1	30 (30.9%)	21.94-41.13
Intraendothelial meronts	0	0	0	0	0	0	1	1 (1.0%)	0.03-5.61
Spleen	*4*	*7*	*35*	*22*	*12*	*2*	*1*	*83*	
Lymphocyte hyperplasia	1	2	4	9	9	0	0	25 (30.1%)	20.53-41.18
Splenitis	0	0	0	0	0	0	1	1 (1.2%)	0.03-6.52
Intraendothelial meronts	0	0	0	0	0	0	1	1 (1.2%)	0.03-6.52

The three most common histopathological findings observed on the wild small mammals were intestinal nematodiasis (32.3%, 21/65, Figure 5.1), lymphoplasmacytic periportal hepatitis (30.9%, 30/97, Figure 5.3) and splenic lymphocyte hyperplasia (30.1%, 25/83, Figure 5.4). Intracytoplasmic apicomplexa parasites in intestinal epithelial cells and cestodes were also observed (Figure 5.2). Intraendothelial meronts were observed in blood vessels of the kidney, liver and spleen of one common shrew (Table 5.1, Figures 5.6 and 5.7); this animal also had lymphoplasmacytic pyelonephritis and splenitis (Figure 5.7B).

Nested PCR for OPV DNA was performed in samples of spleen and/or small intestine of 76 wild small mammals, being eight brown rats, nine house mice, 22 wood mice, 25 bank voles and 12 field voles. The overall prevalence of OPV was 5.8% (12/76, 95% CI 8.44-25.97; Tables 5.2 and 5.3), but it varied considerably between species. As shown on table 5.2, the highest prevalence of OPV infection was observed in field voles (5/12, 41.7%), followed by brown rats (2/8, 25%), wood mouse (3/22, 13.6%), house mouse (1/9, 11.1%) and bank vole (1/25, 4%). Out of

the 12 rodents positive for OPV DNA, eight (66.7%) were trapped in the autumn, and four (33.3%) were trapped in the winter (Table 5.2). Samples collected during the spring and summer were not processed for the presence fo OPV DNA.

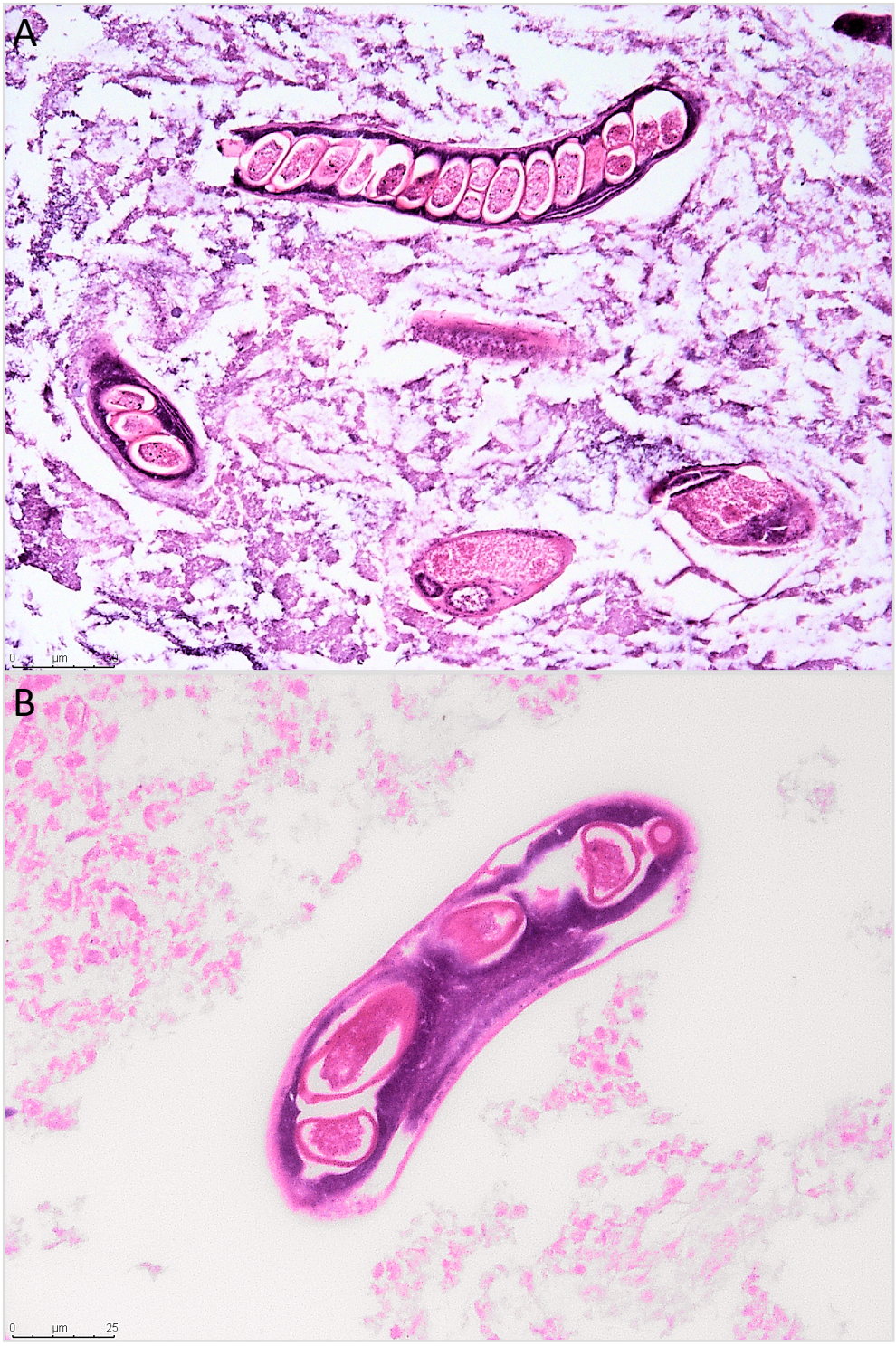

Figure 5.1 - (A and B) Photomicrograph of the small intestine of a field vole with intraluminal adult aphasmid nematodes, with hypodermal bacillary bands and containing eggs with bipolar plugs (A, 20X, scale bar 50µm; B, 40X; HE, scale bar 25µm).

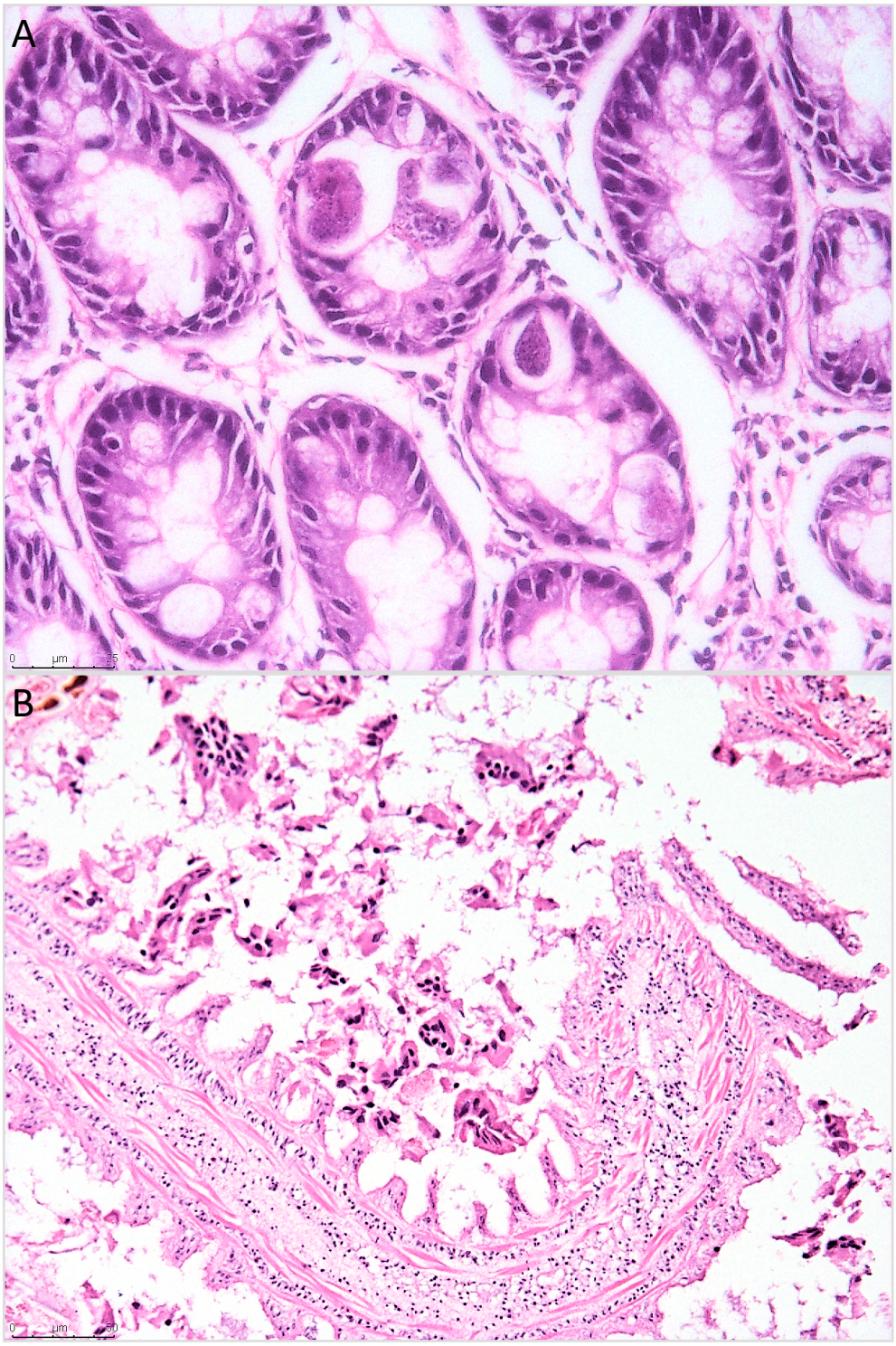

Figure 5.2 -. (A) Photomicrograph of the small intestine of a bank vole with intracytoplasmic apicomplexa parasites in intestinal epithelial cells (40X, scale bar 25µm, HE). (B) Photomicrograph of the small intestine of a bank vole with an intraluminal cestode, showing its segmented body (20X, scale bar 50µm, HE).

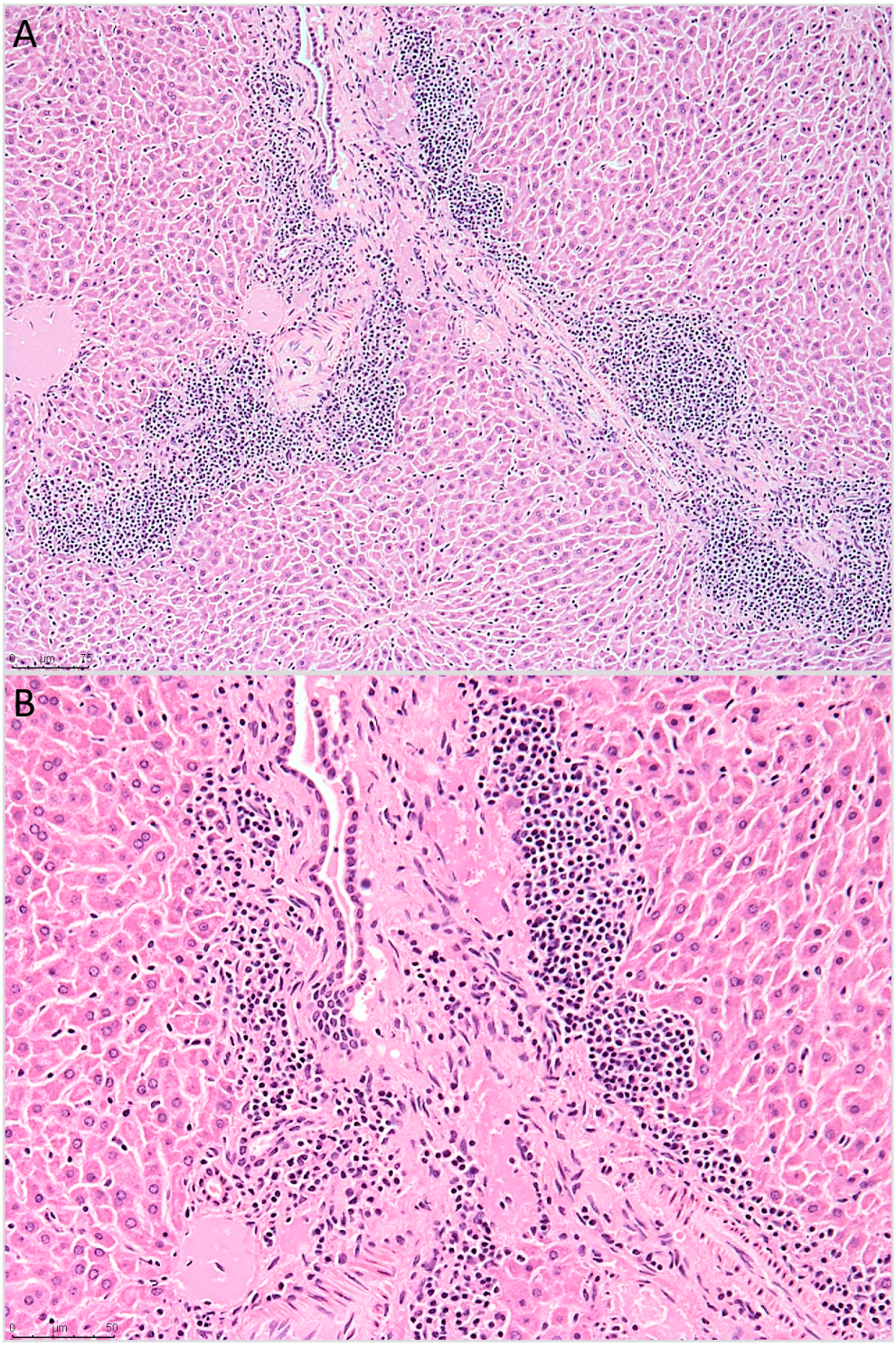

Figure 5.3 - (A) Photomicrograph of the liver of a field vole showing mild to moderate lymphoplasmacytic periportal hepatitis (10X, scale bar 75µm, HE). (B) Closer view of the liver shown in A (20X, scale bar 50µm, HE).

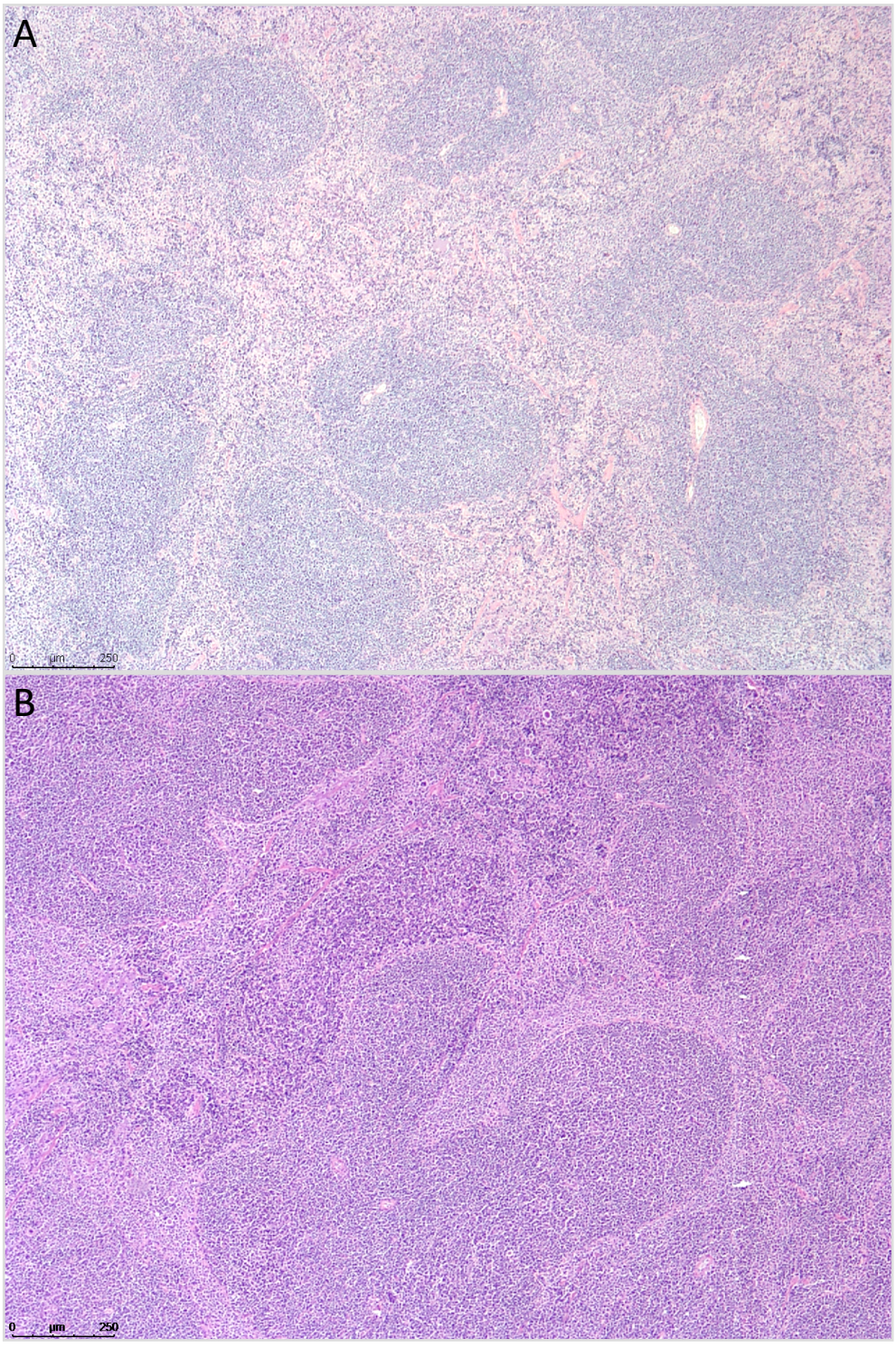

Figure 5.4 - Photomicrograph of the spleen of a field vole (A) and a bank vole (B) with moderate to severe splenic lymphoid hyperplasia (4X, scale bar 250µm, HE).

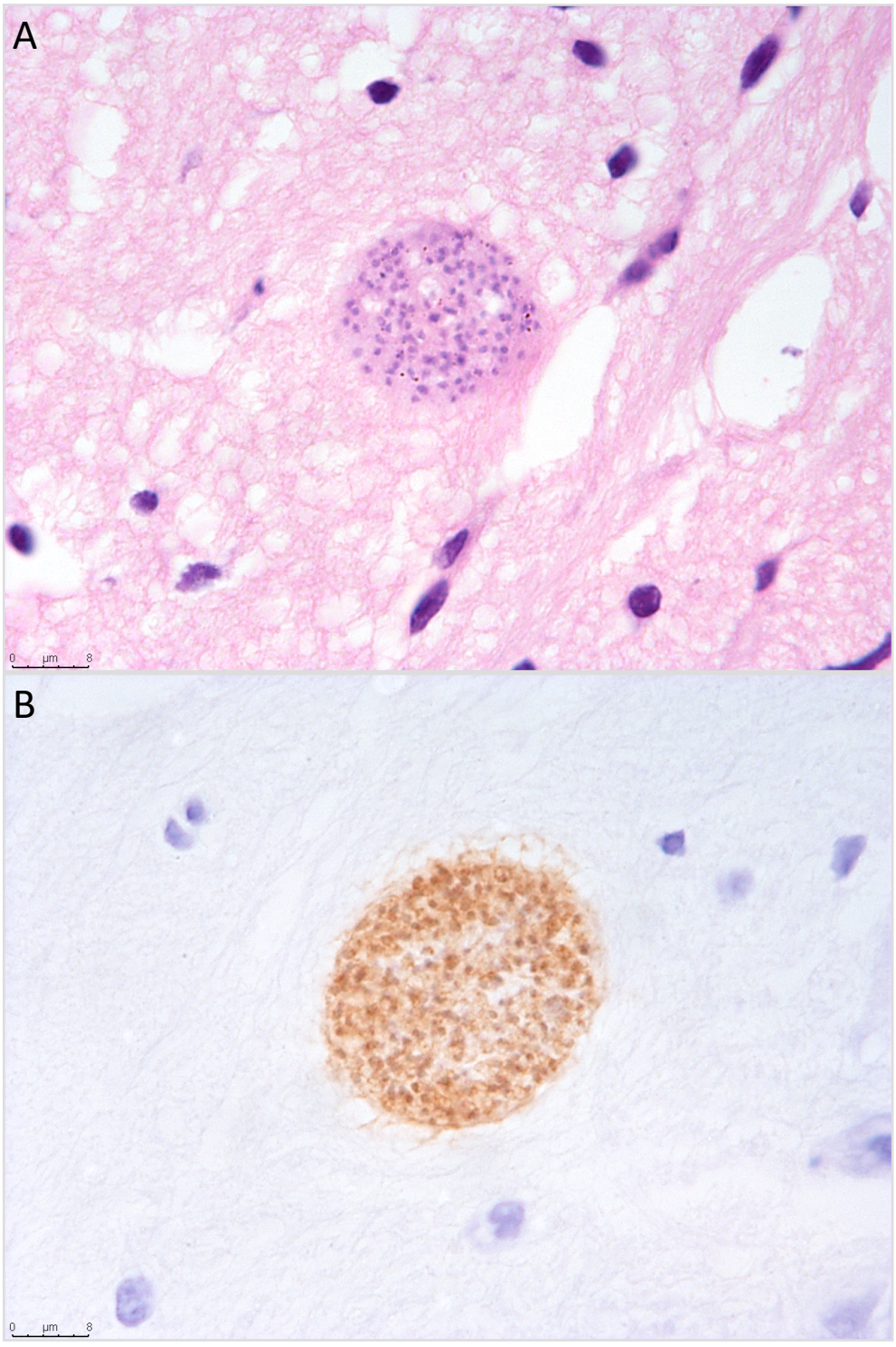

Figure 5.5 - (A) Photomicrograph of the brain of a bank vole showing a tissue cyst containing bradizoites (100X, scale bar 8µm, HE). (B) Immunohistochemical staining to *T. gondii* of the tissue cyst shown in A (100X, scale bar 8µm, IHC to *T. gondii*).

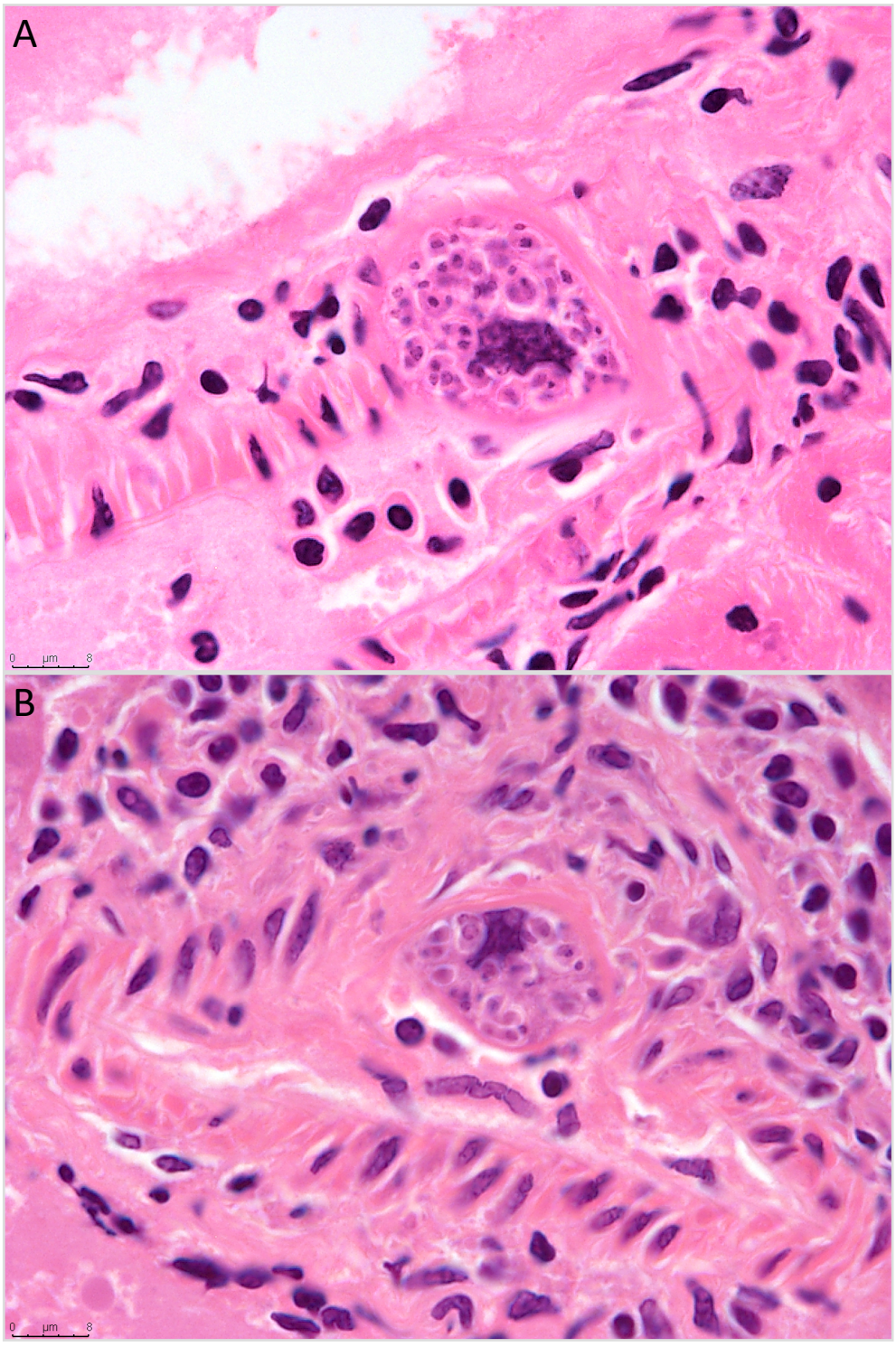

Figure 5.6 - Photomicrographs of the kidney (A, 100X, scale bar 8µm, HE) and liver (B, 100X, scale bar 8µm, HE) of a common shrew, with intracytoplasmic meronts in endothelial cells.

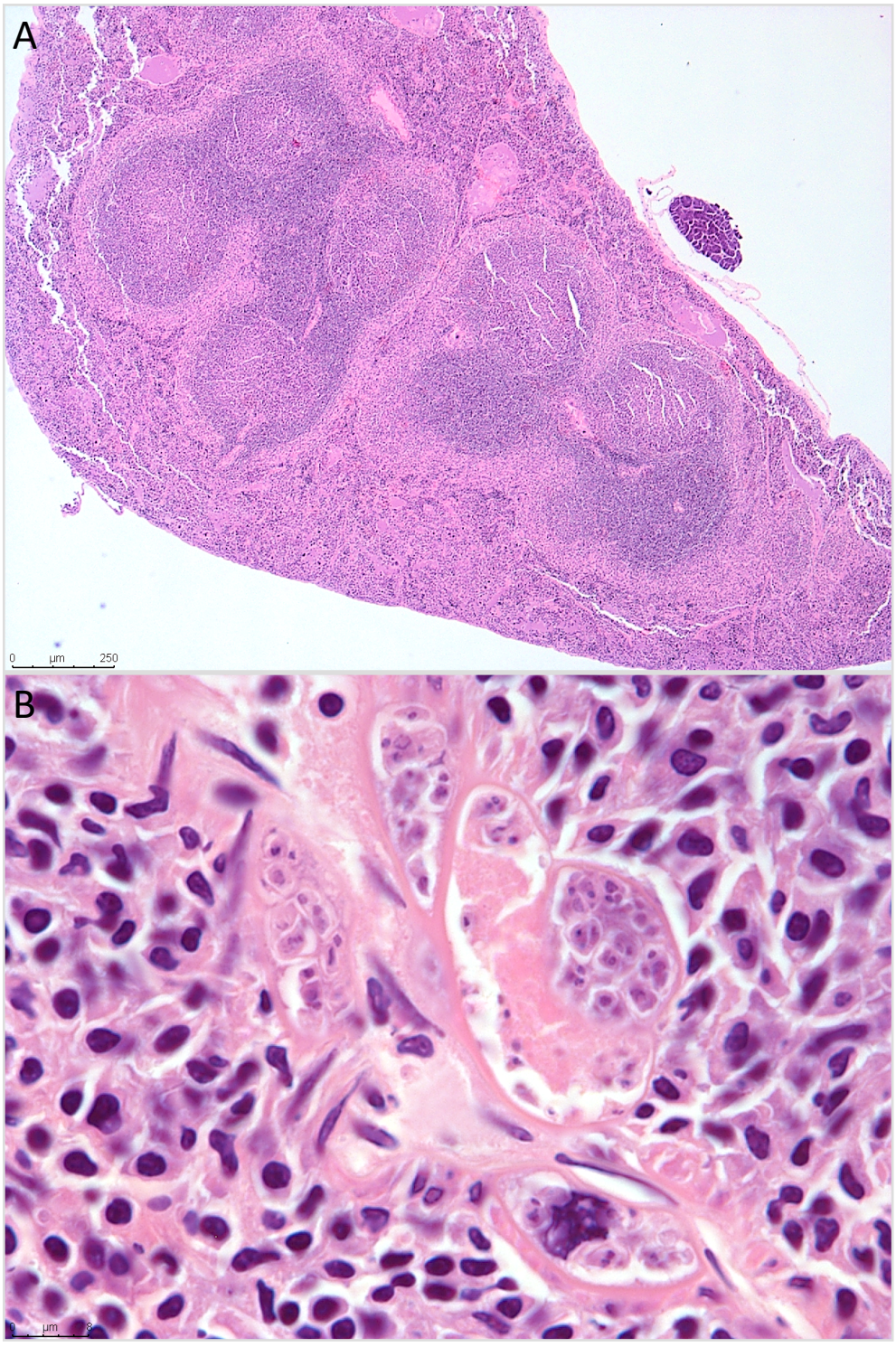

Figure 5.7 - Photomicrographs of the spleen of a common shrew with multifocal to coalescing lymphoplasmacytic splenitis (A, 4X, scale bar 250µm, HE) and intracytoplasmic meronts in endothelial cells (B, 100X, scale bar 8µm, HE).

Table 5.2 - Detection of *Toxoplasma gondii* and *Orthopoxvirus* (OPV) in different wild rodent species trapped in Chester Zoo, from February 2019 to February 2020, by season of the year and trap location.

Season and trap location	Detection of pathogen, by species*						
	T. gondii	*Orthopoxvirus*					
		Number of animals positive to OPV DNA/number of animals tested					
	Number bank voles positive to *T. gondii*/ number of bank voles tested	Brown rat	House mouse	Wood mouse	Bank vole	Field vole	Total
Season (months)							
Winter (Dec-Feb)	0/4	n/a	1/8	0/10	1/5	2/2	4/25
Spring (Mar-May)	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Summer (Jun-Aug)	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Autumn (Sep-Nov)	4/20	2[a]/8	0/1	3/12	0/20	3/10	8/51
Trap location							
Pallas's cat	4/15	n/a	n/a	1/11	1/15	0/2	2/28
Cheetahs	0/5	n/a	n/a	1/9	0/5	3[b]/7	4/21
Jaguars	0/2	n/a	n/a	1[b]/1	0/2	1[b]/2	2/5
Rhinos	n/a	n/a	n/a	0/1	0/1	n/a	0/2
Aye aye	n/a	n/a	1/1	n/a	n/a	n/a	1/1
Realm of the Red Ape	n/a	1/6	0/1	n/a	n/a	n/a	1/5
Capybara pad	n/a	1/1	n/a	n/a	n/a	n/a	1/1
Animal Supplies Department	n/a	0/1	n/a	n/a	n/a	n/a	0/1
June's Pavillion	n/a	n/a	0/1	n/a	n/a	n/a	0/1
Free Flight Aviary	n/a	n/a	0/1	n/a	n/a	n/a	0/1
Parrots	n/a	n/a	0/4	n/a	n/a	n/a	0/4
Caughall Farm	n/a	n/a	0/1	n/a	n/a	n/a	0/1
Unknown	0/2	n/a	n/a	n/a	0/2	1[b]/1	1/3
Total	*4/24*	*2/8*	*1/9*	*3/22*	*1/25*	*5/12*	*12/76*
Prevalence	*16.7%*	*25%*	*11.1%*	*13.6%*	*4%*	*41.7%*	*15.8%*
95% CI (%)	*4.75-37.42*	3.19-65.09	0.28-48.25	2.9-34.9	0.1-20.35	15.2-72.4	*8.4-25.9*

*Positivity to *T. gondii* was determined by the presence of positive IHC staining in the brain; positivity to *Orthopoxvirus* was determined by the presence of OPV DNA in the spleen and/or small intestine using a nested PCR.
[a]The two brown rats were positive for the presence of OPV DNA in both the small intestine and spleen.
[b]2/3 wood mice and 4/5 field voles were weakly positive for the presence of OPV DNA (tested positive in only one of the triplicate nested PCR reaction tube).
n/a, not applicable; CI, confidence interval.

Discussion

This disease surveillance study demonstrated that OPV circulates in multiple species of wild rodents in Chester Zoo: brown rat, house mouse, wood mouse, bank vole, and field vole. The species with the highest prevalence of OPV infection was bank vole, suggesting that they might be more likely implicated in the epidemiology of OPV transmission to zoo animals at Chester Zoo than other rodent species.

Previous studies have suggested that wild brown rats are the most probable source of CPXV to non-domestic animals (Martina et al., 2006); similarly, our study showed a relatively high prevalence of OPV DNA in brown rats at Chester Zoo (25%). And although our results might suggest that these animals could play an important role on the epidemiology of this disease, the number of animals tested for the presence of OPV DNA was very small (n=8) and, therefore, these results should be interpreted with caution. Further studies with high number of individuals are needed in order to fully understand the role of brown rats on the epidemiology of OPV infection in zoo animals.

Although the overall prevalence of toxoplasmosis in wild small mammals was low (4/109, 3.7%), a moderate prevalence (4/24, 16.7%) of toxoplasmosis was observed in bank voles. All four bank voles with toxoplasmosis were trapped by the Pallas's cat enclosure. Interestingly, a fatal outbreak of toxoplasmosis in Pallas's cats occurred in this enclosure in 2018 (Costa et al., 2020). Taken together, these findings suggest that *T. gondii* is still circulating and that bank voles might be an important reservoir of toxoplasmosis for animals housed in this enclosure. However, due to the small number of brain samples, it is not possible to rule out the implication of other rodent species in the transmission of toxoplasmosis to zoo animals in Chester Zoo.

Periportal lymphoplasmacytic hepatitis was observed in five species, with higher incidence in field voles and bank voles. Similar histopathological lesions are observed in wild rodents infected with hepatitis E virus (Murphy et al., 2019). Further studies are encouraged to determine the cause of the hepatitis observed in these wild rodents. Similarly, a high indigence of splenic lymphocyte hyperplasia was observed; this is an unspecific histopathological lesion that indicates a reactive splenic lymphoid tissue to infection.

One out of the three common shrews examined histologically had a systemic protozoal infection, characterised by multi-systemic intraendothelial meronts. It is not possible to accurately determine the species of protozoa implicated in this case; however, the lack of inflammatory response associated with the presence of intraendothelial meronts suggest that this is an incidental finding of no clinical significance for the common shrew.

A high number of tissue samples were not suitable for histopathological analysis due to extensive post-mortem autolysis. Moreover, many of the samples had freezing artefacts. These changes are due to the animal trapping and collection methods used in this research project (lethal traps being checked after 14-16h; carcasses frozen until PME was performed). These methods were deliberately chosen for a two reasons: first, lethal trapping is the current method used for pest management control at Chester Zoo, and it has been approved by the Chester Zoo's ethical committee; second, freezing the carcases after collection was a readily available method of sample storage and would not require extensive logistical planning. And although live trapping, followed by euthanasia (e.g. isofluorane general anaesthesia followed by intracardiac overdose of barbiturate, or cervical dislocation) and PME would have resulted in no to minimal post-mortem autolysis and avoided freezing artefacts, it would have required a much more extensive planning and logistics, including further ethical approval (from both Chester Zoo and the University of Liverpool), handling and transporting of live animals, risk assessments in place and immediate availability for post-mortem examination. Moreover, due to limitations in time and financial resources, it was not possible to perform PME, histopathology, immunohistochemistry and OPV DNA PCR on all animals and samples collected. Ideally, not only we would have processed all the animals and samples collected, but we would have tested them for other pathogens that could be important for zoo animals and/or humans, such as hepatitis E virus, hantavirus, lymphocytic choriomeningitis virus, and *Yersinia pseudotuberculosis*.

In conclusion, wild rodents at Chester Zoo do carry pathogens which could pose a significant risk of infection, including cowpox and toxoplasmosis, not only to zoo animals, but also to personnel involved in the care of these animals. Biosecurity and pest control measures must be implemented, encouraged and/or enforced in order to reduce the chances of zoo animals or staff members getting in contact with wild rodents, preventing future outbreaks of rodent-associated diseases.

6. GENERAL DISCUSSION AND FINAL CONCLUSIONS

Zoological collections have a duty of care to provide animal healthcare to their collections, and to protect the health, safety and welfare of their employees and visitors, including protection from infectious zoonotic diseases. And this is achieved with a combination of actions, including biosecurity measures and investigation of morbidity and mortality events. Zoological collections suffer from sporadic outbreaks of infectious diseases, and many of them are rodent-associated diseases, such as cowpox and toxoplasmosis.

The incidence of cowpox in both humans and animals in Europe has risen in recent years, leading CPXV to be considered an emerging public health threat. Although cases and outbreaks of cowpox have been extensively studied, aspects of the epidemiology and pathobiology of this disease vary between regions and are not fully understood. The overall goal of this research project was to contribute to the diagnostic capacity of the group and to a better understanding of the epidemiology of cowpox infection in non-domestic animals and its wild reservoirs.

This study improved the capabilities of the Veterinary Pathology Diagnostic Service of the University of Liverpool to diagnose OPV infections. This was successfully achieved by the development of an IHC protocol to identify OPV antigen in FFPE tissues (Chapter 3). This technique was shown to work successfully in tissues from non-domestic animals, such as cheetahs, snow leopards, Chilean pudus, cotton-top tamarins, Goeldi's monkeys, red pandas, and giant anteaters, as well as domestic cats (Chapters 3 and 4). The Veterinary Pathology Diagnostic Service of the University of Liverpool is now able to offer this technique as part of their routine IHC protocols, contributing to a more accurate diagnosis of OPV infections in domestic animals, and, ultimately, to the animal healthcare and welfare of zoological collections.

The use of CPXV specific monoclonal antibody to detect CPXV antigen in FFPE tissues was considered in the early stages of this study, as it has been successfully used by other authors (Schaudien et al., 2007; Herder et al., 2011). This antibody would have allowed the immunohistochemical confirmation of CPXV as the species of OPV involved in the cases presented here. However, as such antibody is not commercially available, anti-vaccinia virus antibodies targeted to the OPV A27L fusion protein were chosen.

Although multiple reports of OPV and CPXV cases and outbreaks in zoo animals have been reported, clinical disease is underreported. This study compiled epidemiological information of previously unpublished cases of OPV and CPXV infections in non-domestic animals in the United Kingdom, and reported for the first time clinical disease in snow leopard, Chilean pudu, aardvark, and Malayan tapir

(Chapter 4). We acknowledge that this study does not represent the actual number of affected animals in each outbreak, as samples from affected conspecifics are not necessary submitted for diagnostic confirmation. Moreover, as this study is based on samples submitted to diagnostic pathology services, it is biased towards fatal OPV cases. Our results indicate an even wider and more varied range of non-domestic animals susceptible to OPV than those previously reported. This study contributes to a better understanding of the epidemiology of OPV, particularly the wide range of species susceptible to this infection. Further case reports of OPV and CPXV in non-domestic animals are encouraged.

Last, but not least, this study investigated the prevalence of OPV and toxoplasmosis in wild small mammals from Chester Zoo. The results showed a high prevalence of OPV DNA in brown rats (2/8, 25%) and a moderate prevalence (4/24, 16.7%) of toxoplasmosis in bank voles. However, these prevalences are relative and must be interpreted with caution. For instance, only eight brown rats were tested for the presence of OPV DNA, and the overall prevalence of toxoplasmosis in wild small mammals was low (4/109, 3.7%). Nevertheless, this study shows strong evidence that OPV and *T. gondii* circulate in wild small mammals in Chester Zoo (Chapter 5). These findings highlight the importance of stringent biosecurity measures and pest management control in zoological collections, in order to prevent or reduce the chances of OPV and toxoplasmosis transmission between wild rodents and zoo animals or humans occurring.

The number of samples processed in this study were reduced due to limitations in time and resources. Nevertheless, this study was able to demonstrate the role of wild rodents as carriers of relevant pathogens to zoo animals and personnel, as well as the importance of rodent health surveillance and stringent biosecurity measures in zoological collections.

The OPV nested PCR used in this study (Chapters 4 and 5) is aimed at the conserved 14KDa OPV fusion protein gene, and does not allow differentiation between OPVs. Consequently, and despite many of the cases on having classical macroscopic and histological CPXV lesions, we were limited in the conclusions we could draw, and the cases were diagnosed as OPV infections only. It would have been extremely useful to perform more specific molecular diagnostic assays, such as real-time PCR or sequencing and phylogenetic analysis of the samples. Such techniques would have allowed further characterisation of the OPV involved and the assessment of the phylogenetic relationship between the isolates.

Further research is needed to elucidate some questions raised in this study. For instance, it would be interesting to clarify the exact role of brown rats in the epidemiology of OPV. This could be achieved with a more extensive surveillance study of brown rats and the processing of a higher number of samples for

histopathology and for the presence of OPV or CPXV DNA, or the use of experimental infections in brown rats. Moreover, further investigation is encouraged to determine the cause of the periportal lymphoplasmacytic hepatitis observed in wild small mammals trapped at Chester Zoo.

Taken together, the results of this study improved the diagnostic ability of OPV infection and has shown that the range of non-domestic animals susceptible to this infection is even wider than previously thought. It also shown that multiple wide rodents species might be infected with and implicated on the transmission cycle of OPV in the United Kingdom. Ultimately, this information helps to achieve a higher degree of animal healthcare and welfare in zoological collections, and highlights the importance of biosecurity measures. Moreover, this study provides tools and baseline data that can benefit future diagnostic and research trials with non-domestic animals and wild rodents.

7. REFERENCES

Ashpole, I. P., Chantrey, J., Lopez, J., Drake, G. and Steinmetz, H. W. (2020). Successful treatment of clinical Orthopoxvirus infection in a giant anteater (*Myrmecophaga tridactyla*). *Journal of Zoo and Wildlife Medicine,* **51,** 217-221.

Basse, A., Freundt, E. and Hansen, F. (1964). Ein Ausbruch von Pockenkrankheit bei Okapis im Kopenhagener Zoo. *Verh ber Erkrg Zootiere,* **6,** 55-62.

Baxby, D. (1977). Is cowpox misnamed? A review of 10 human cases. *British Medical Journal,* **1,** 1379-1381.

Baxby, D. (1993). Indications for smallpox vaccination: policies still differ. *Vaccine,* **11,** 395-396.

Baxby, D., Ashton, D. G., Jones, D. M. and Thomsett, L. R. (1982). An outbreak of cowpox in captive cheetahs: virological and epidemiological studies. *Journal of Hygiene,* **89,** 365-372.

Baxby, D., Bennett, M. and Getty, B. (1994). Human cowpox 1969-93: a review based on 54 cases. *British Journal of Dermatology,* **131,** 598-607.

Baxby, D. and Ghaboosi, B. (1977). Laboratory characteristics of poxviruses isolated from captive elephants in Germany. *Journal of General Virology,* **37,** 407-414.

Baxby, D., Shackleton, W. B., Wheeler, J. and Turner, A. (1979). Comparison of cowpox-like viruses isolated from European zoos. *Archives of Virology,* **61,** 337-340.

Becker, C., Kurth, A., Hessler, F., Kramp, H., Gokel, M., Hoffmann, R., Kuczka, A. and Nitsche, A. (2009). Cowpox virus infection in pet rat owners: not always immediately recognized. *Deutsches Aerzteblatt International,* **106,** 329-334.

Bennett, M. (2014). Feline poxvirus infections. In: *Canine and feline infectious diseases*, J. E. Sykes, Ed, Elsevier Saunders, Missouri, pp. 252-256.

Bennett, M. and Baxby, D. (1996). Cowpox. *Journal of Medical Microbiology,* **45,** 157-158.

Bennett, M., Gaskell, R. M., Gaskell, C. J., Baxby, D. and Kelly, D. F. (1989). Studies on poxvirus infection in cats. *Archives of Virology,* **104,** 19.

Bernard, M. (2012). Poxvirus cell entry: how many proteins does it take? *Viruses,* **4,** 688-707.

Bonnekoh, B., Falk, K., Reckling, K.-F., Kenklies, S., Nitsche, A., Ghebremedhin, B., Pokrywka, A., Franke, I., Thriene, B., König, W., Pauli, G. and Gollnick, H. (2008). Cowpox infection transmitted from a domestic cat. *Journal of the German Society of Dermatology,* **6,** 210-213.

Breheny, C. R., Fox, V., Tamborini, A., O'Halloran, C., Robertson, E., Cazzini, P., Birn-Jeffery, D., Henkin, J., Schwartz, T., Scase, T., Powell, R. and Gunn-Moore, D.

(2017). Novel characteristics identified in two cases of feline cowpox virus infection. *Journal of Feline Medicine and Surgery Open Reports,* **3,** 1-5.

Cardeti, G., Brozzi, A., Eleni, C., Polici, N., D'Alterio, G., Carletti, F., Scicluna, M. T., Castilletti, C., Capobianchi, M. R., Di Caro, A., Autorino, G. L. and Amaddeo, D. (2011). Cowpox virus in llama, Italy. *Emerging Infectious Diseases,* **17,** 1513-1515.

Carroll, D. S., Emerson, G. L., Yu, L., Sammons, S., Olson, V., Frace, M., Nakazawa, Y., Czerny, C. P., Tryland, M., Kolodziejek, J., Nowotny, N., Olsen-Rasmussen, M., Khristova, M., Govil, D., Karem, K., Damon, I. K. and Meyer, H. (2011). Chasing Jenner's Vaccine: Revisiting Cowpox Virus Classification. *Plos One,* **6,** e23086.

Cartun, R., Taylor, C. and Dabbs, D. (2018). Techniques of immunohistochemistry: principles, pitfalls, and standardization. In: *Diagnostic immunohistochemistry: theranostic and genomic applications*, D. Dabbs, Ed, Elsevier, Philadelphia, pp. 1-46.

Chantrey, J., Meyer, H., Baxby, D., Begon, M., Bown, K. J., Hazel, S. M., Jones, T., Montgomery, W. I. and Bennett, M. (1999). Cowpox: reservoir hosts and geographic range. *Epidemiology and Infection,* **122,** 455-460.

Chi-Long, L., Che-Sheng, C., Heine, H. G. and Chang, W. (2000). Vaccinia virus envelope H3L protein binds to cell surface heparan sulfate and is important for intracellular mature virion morphogenesis and virus infection*in vitro*and*in vivo*. *Journal of Virology,* **74,** 3353–3365.

Chiu, W. L., Lin, C. L., Yang, M. H., Tzou, D. L. M. and Chang, W. (2007). Vaccinia virus 4c (A26L) protein on intracellular mature virus binds to the extracellular cellular matrix laminin. *Journal of Virology,* **81,** 2149-2157.

Chomel, B. B. (2014). Emerging and re-emerging zoonoses of dogs and cats. *Animals (Basel),* **4,** 434-445.

Chung, C. S., Hsiao, J. C., Chang, Y. S. and Chang, W. (1998). A27L protein mediates vaccinia virus interaction with cell surface heparan sulfate. *Journal of Virology,* **72,** 1577-1585.

Coras, B., Eßbauer, S., Pfeffer, M., Meyer, H., Schroöder, J., Stolz, W., Landthaler, M. and Vogt, T. (2005). Cowpox and a cat. *Lancet,* **365,** 446-446.

Costa, T., Ashpole, I. and Chantrey, J. (2020). Fatal outbreak of toxoplasmosis in captive Pallas's cats (*Otocolobus manul*). *Journal of Comparative Pathology,* **174,** 174.

Cregger, M., Berger, A. J. and Rimm, D. L. (2006). Immunohistochemistry and quantitative analysis of protein expression. *Archives of Pathology and Laboratory Medicine,* **130,** 1026-1030.

Crouch, A. C., Baxby, D., McCracken, C. M., Gaskell, R. M. and Bennett, M. (1995). Serological Evidence for the Reservoir Hosts of Cowpox Virus in British Wildlife. *Epidemiology and Infection,* **115,** 185.

Cudmore, S., Cossart, P., Griffiths, G. and Way, M. (1995). Actin-based motility of vaccinia virus. *Nature,* **378,** 636-638.

Czerny, C.-P., Eis-Hübinger, A. M., Mayr, A., Schneweis, K. E. and Pfeiff, B. (1991). Animal poxviruses transmitted from cat to man: current event with lethal end. *Journal of Veterinary Medicine, Series B,* **38,** 421-431.

Dabrowski, P. W., Radonić, A., Kurth, A. and Nitsche, A. (2013). Genome-wide comparison of cowpox viruses reveals a new clade related to Variola virus. *Plos One,* **8,** e79953.

Damon, I. (2007). Poxviruses. In: *Fields Virology*, D. Knipe and P. Howley, Eds, Vol. 2, Lippincott Williams & Wilkins, Philadelphia, pp. 2947-2975.

DEFRA. (2012). Secretary of State's Standards of Modern Zoo Practice, DEFRA, Ed, pp. 1-90.

Downie, A. W. (1939). Immunological relationship of the virus of spontaneous cowpox to vaccina virus. *British Journal of Experimental Pathology,* **20,** 158-176.

Dubey, J. P. (2010). Toxoplasmosis in domestic cats and other felids. In: *Toxoplasmosis of animals and humans*, J. P. Dubey, Ed, CRC Press, Boca Raton, pp. 95-118.

Dubey, J. P. and Dubey, J. P. Toxoplasmosis of animals and humans. CRC Press.

Duraiyan, J., Govindarajan, R., Kaliyappan, K. and Palanisamy, M. (2012). Applications of immunohistochemistry. *Journal of Pharmacy and Bioallied Sciences,* **4,** S307-S309.

Elsendoorn, A., Agius, G., Le Moal, G., Aajaji, F., Favier, A.-L., Wierzbicka-Hainault, E., Béraud, G., Flusin, O., Crance, J.-M. and Roblot, F. (2011). Severe ear chondritis due to cowpox virus transmitted by a pet rat. *Journal of Infection,* **63,** 391-393.

Esposito, J. J. and Knight, J. C. (1985). Orthopoxvirus DNA: A comparison of restriction profiles and maps. *Virology,* **143,** 230-251.

Esposito, J. J., Sammons, S. A., Frace, A. M., Osborne, J. D., Olsen-Rasmussen, M., Zhang, M., Govil, D., Damon, I. K., Kline, R., Laker, M., Li, Y., Smith, G. L., Meyer, H., LeDuc, J. W. and Wohlhueter, R. M. (2006). Genome sequence diversity and clues to the evolution of variola (smallpox) virus. *Science,* **313,** 807-812.

Essbauer, S., Pfeffer, M. and Meyer, H. (2010). Zoonotic poxviruses. *Veterinary Microbiology,* **140,** 229-236.

Eulenberger, K., Berhard, A., Nieper, H., Hoffmann, K., Scheller, R., Meyer, H., Zimmerman, P., Essbauer, S., Pfeffer, M. and Kiessling, J. (2005). An outbreak of cowpox infection in a black rhino (*Diceros bicornis*) at Leipzig Zoo. *Erkrankungen*

der Zootiere: Verhandlungsbericht des Internationalen Symposiums über die Erkrankungen der Zoo- und Wildtiere, Prague, Czech Republic, May 04-08, 2005, pp. 77-85.

Fenner, F., Henderson, D. A., Arita, I., Jezek, Z. and Ladnyi, I. D. (1988). *Smallpox and its eradication*. World Health Organization Geneva.

Fischer, S., Spierling, N. G., Ulrich, R. G., Franke, A., Beer, M., Hoffmann, D., Imholt, C., Jacob, J. and Gethmann, J. (2020). Patchy occurrence of cowpox virus in voles from Germany. *Vector-Borne and Zoonotic Diseases,* **20,** 471-475.

Franke, A., Kershaw, O., Jenckel, M., König, L., Beer, M., Hoffmann, B. and Hoffmann, D. (2016). Fatal cowpox virus infection in an aborted foal. *Vector-Borne and Zoonotic Diseases,* **16,** 431-433.

Franke, A., Pfaff, F., Jenckel, M., Hoffmann, B., Höper, D., Antwerpen, M., Meyer, H., Beer, M. and Hoffmann, D. (2017). Classification of cowpox viruses into several distinct clades and identification of a novel lineage. *Viruses,* **9,** 142.

Gavrilova, E. V., Shcherbakov, D. N., Maksyutov, R. A. and Shchelkunov, S. N. (2010). Development of real-time PCR assay for specific detection of cowpox virus. *Journal of Clinical Virology,* **49,** 37-40.

Gazzani, P., Gach, J. E., Colmenero, I., Martin, J., Morton, H., Brown, K. and Milford, D. V. (2017). Fatal disseminated cowpox virus infection in an adolescent renal transplant recipient. *Pediatric Nephrology (Berlin, Germany),* **32,** 533-536.

Gehring, H., Mahnel, H. and Mayer, H. (1972). Elefantenpocken. *Zentralblatt für Veterinärmedizin Reihe B,* **19,** 258-261.

Girling, S. J., Pizzi, R., Cox, A. and Beard, P. M. (2011). Fatal cowpox virus infection in two squirrel monkeys (*Saimiri sciureus*). *Veterinary Record,* **169,** 156.

Godfrey, D. R., Blundell, C. J., Essbauer, S., Pfeffer, M., Shearer, D. H., Rest, J. R. and Baker, J. F. M. (2004). Unusual presentations of cowpox infection in cats. *Journal of Small Animal Practice,* **45,** 202-205.

Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P. and Paoletti, E. (1990). The complete DNA sequence of vaccinia virus. *Virology,* **179,** 247-266.

Grimm, C., Hillen, H. S., Bedenk, K., Bartuli, J., Neyer, S., Zhang, Q., Hüttenhofer, A., Erlacher, M., Dienemann, C., Schlosser, A., Urlaub, H., Böttcher, B., Szalay, A. A., Cramer, P. and Fischer, U. (2019). Structural basis of poxvirus transcription: vaccinia RNA polymerase complexes. *Cell,* **179,** 1537-1550.

Grönemeyer, L.-L., Baltzer, A., Broekaert, S., Schrick, L., Möller, L., Nitsche, A., Mössner, R., Schön, M. P. and Buhl, T. (2017). Generalised cowpox virus infection. *Lancet,* **390,** 1769.

Grzybek, M., Tołkacz, K., Sironen, T., Mäki, S., Alsarraf, M., Behnke-Borowczyk, J., Biernat, B., Nowicka, J., Vaheri, A., Henttonen, H., Behnke, J. M. and Bajer, A. (2020). Zoonotic viruses in three species of voles from Poland. *Animals (Basel),* **10**.

Guthrie, A., Rooker, L., Tan, R., Gerhold, R., Trainor, K., Jiang, T. and Su, C. (2017). Newly described*Toxoplasma gondii*strain causes high mortality in red-necked wallabies (*Macropus rufogriseus*) in a zoo. *Journal of Zoo and Wildlife Medicine,* **48,** 694-702.

Haase, O., Moser, A., Rose, C., Kurth, A., Zillikens, D. and Schmidt, E. (2011). Generalized cowpox infection in a patient with Darier disease. *British Journal of Dermatology,* **164,** 1116-1118.

Haddadeen, C., Van Ouwerkerk, M., Vicek, T. and Fityan, A. (2020). A case of cowpox virus infection in the UK occurring in a domestic cat and transmitted to the adult male owner. *British Journal of Dermatology,* **183,** e190.

Hemmer, C. J., Littmann, M., Lobermann, M., Meyer, H., Petschaelis, A. and Reisinger, E. C. (2010). Human cowpox virus infection acquired from a circus elephant in Germany. *International Journal of Infectious Disease,* **14 Suppl 3,** e338-340.

Hentschke, J., Meyer, H., Wittstatt, U., Ochs, A., Burkhardt, S. and Aue, A. (1999). Kuhpocken bei kanadischen bibern (*Castor fiber canadensis*) und Katzenbären (*Ailurus fulgens*). *Tierarztliche Umschau,* **54,** 311-317.

Herder, V., Wohlsein, P., Grunwald, D., Janssen, H., Meyer, H., Kaysser, P., Baumgärtner, W. and Beineke, A. (2011). Poxvirus infection in a cat with presumptive human transmission. *Veterinary Dermatology,* **22,** 220-224.

Hinrichs, U., van de Poel, H. and van den Ingh, T. S. (1999). Necrotizing pneumonia in a cat caused by an orthopox virus. *Journal of Comparative Pathology,* **121,** 191-196.

Hoffmann, D., Franke, A., Jenckel, M., Schluckebier, J., Hoffmann, B., Höper, D., Goller, K., Beer, M., Tamošiunaite, A., Osterrieder, N., Granzow, H., Fischer, S. and Ulrich, R. G. (2015). Out of the reservoir: Phenotypic and genotypic characterization of a novel cowpox virus isolated from a common vole. *Journal of Virology,* **89,** 10959-10969.

Hsiao, J. C., Chung, C. S. and Chang, W. (1998). Cell surface proteoglycans are necessary for A27L protein-mediated cell fusion: Identification of the N-terminal region of A27L protein as the glycosaminoglycan-binding domain. *Journal of Virology,* **72,** 8374-8379.

Hsiao, J. C., Chung, C. S. and Chang, W. (1999). Vaccinia virus envelope D8L protein binds to cell surface chondroitin sulfate and mediates the adsorption of intracellular mature virions to cells. *Journal of Virology,* **73,** 8750-8761.

Ichihashi, Y., Matsumoto, S. and Dales, S. (1971). Biogenesis of poxviruses: Role of A-type inclusions and host cell membranes in virus dissemination. *Virology,* **46,** 507-532.

Jenner, E. (1789). An inquiry into the causes and effects of the variolæ vaccinæ, a disease discovered in some of the western counties of England, particularly Gloucestershire, and known by the name of the cow pox. Sampson Low, London.

Jenner, E. (1798). An inquiry into the causes and effects of the variolæ vaccinæ, a disease discovered in some of the western counties of England, particularly Gloucestershire, and known by the name of the cow pox. Sampson Low, London, pp. 182.

Jeske, K., Weber, S., Pfaff, F., Imholt, C., Jacob, J., Beer, M., Ulrich, R. G. and Hoffmann, D. (2019). Molecular detection and characterization of the first cowpox virus isolate derived from a bank vole. *Viruses,* **11,** 1075.

Jungwirth, N., Puff, C., Köster, K., Mischke, R., Meyer, H., Stark, A., Thoma, B., Zöller, G., Seehusen, F., Hewicker-Trautwein, M., Beineke, A., Baumgärtner, W. and Wohlsein, P. (2018). Atypical Cowpox Virus Infection in a Series of Cats. *Journal of Comparative Pathology,* **158,** 71-76.

Kiernan, M. and Koutroumanos, N. (2021). Orbital cowpox. *New England Journal of Medicine,* **384,** 2241.

Kik, M. J. L., Liu, P. L. and van Asten, J. A. M. (2006). Cowpoxvirus infection in the Patagonian cavy (*Dolichotis patagonum*) emerging disease in an educational animal park the first reported case. *Veterinary Quarterly,* **28,** 42-44.

Krankowska, D. C., Woźniak, P. A., Cybula, A., Izdebska, J., Suchacz, M., Samelska, K., Wiercińska-Drapało, A. and Szaflik, J. P. (2021). Cowpox: how dangerous could it be for humans? Case report. *International Journal of Infectious Diseases,* **104,** 239-241.

Krijnse Locker, J., Kuehn, A., Schleich, S., Rutter, G., Hohenberg, H., Wepf, R. and Griffiths, G. (2000). Entry of the two infectious forms of vaccinia virus at the plasma membane is signaling-dependent for the IMV but not the EEV. *Molecular Biology of the Cell,* **11,** 2497-2511.

Kubin, G., Koelbl, O. and Gerstl, F. (1975). Charakterisierung eines vom Elefanten isolierten Pockenvirusstammes. *Wien Tieraerztl Mschr,* **62,** 271-276.

Kurth, A. and Nitsche, A. (2007). Fast and reliable diagnostic methods for the detection of human poxvirus infections. *Future Virology,* **2,** 467-479.

Kurth, A. and Nitsche, A. (2012). Cowpox in zoo animals. In: *Fowler's zoo and wild animal medicine current therapy*, R. Miller and M. Fowler, Eds, Vol. 7, Saunders, St. Louis, pp. 32-37.

Kurth, A., Pauli, G., Nitsche, A., Wibbelt, G., Gerber, H. P. and Petschaelis, A. (2008). Rat-to-elephant-to-human transmission of cowpox virus. *Emerging Infectious Diseases,* **14,** 670-671.

Kurth, A., Straube, M., Kuczka, A., Dunsche, A. J., Meyer, H. and Nitsche, A. (2009). Cowpox Virus Outbreak in Banded Mongooses (Mungos mungo) and Jaguarundis (Herpailurus yagouaroundi) with a Time-Delayed Infection to Humans. *Plos One,* **4,** 1-10.

L'Vov, S. D., Gromashevskiĭ, V. L., Marennikova, S. S., Bogoiavlenskiĭ, G. V. and Baĭluk, F. N. (1988). [Isolation of poxvirus (Poxviridae, Orthopoxvirus, the cowpox complex) from the root vole*Microtus (M.) oeconomus*Pal. 1776 in the forest tundra of the Kola Peninsula]. *Voprosy Virusologii,* **33,** 92-94.

Ladnyi, I. D., Ogorodnikova, Z. I., Shelukhina, E. M., Gerasimenko, R. T. and Voronin, Y. S. (1975). A sudy of poxvirus occurence in animals. *Problemy osobo opasnykh Infektsii,* **3-4,** 165-167.

Lawn, R. (2010). Risk of cowpox to small animal practitioners. *Veterinary Record,* **166,** 631.

Li, Y., Meyer, H., Zhao, H. and Damon, I. K. (2010). GC content-based pan-pox universal PCR assays for poxvirus detection. *Journal of Clinical Microbiology,* **48,** 268-276.

Lin, F. and Chen, Z. (2014). Standardization of diagnostic immunohistochemistry: literature review and geisinger experience. *Archives of Pathology and Laboratory Medicine,* **138,** 1564-1577.

Loparev, V. N., Massung, R. F., Esposito, J. J. and Meyer, H. (2001). Detection and differentiation of Old World orthopoxviruses: Restriction fragment length polymorphism of the crmB gene region. *Journal of Clinical Microbiology,* **39,** 94-100.

Magaki, S., Hojat, S. A., Wei, B., So, A. and Yong, W. H. (2019). An introduction to the performance of immunohistochemistry. *Methods in Molecular Biology,* **1897,** 289-298.

Maksyutov, R. A., Gavrilova, E. V., Meyer, H. and Shchelkunov, S. N. (2015). Real-time PCR assay for specific detection of cowpox virus. *Journal of Virological Methods,* **211,** 8-11.

Marennikova, S. S., Maltseva, N. N., Korneeva, V. I. and Garanina, N. M. (1977). Outbreak of pox disease among Carnivora (Felidae) and edentata. *Journal of Infectious Diseases,* **135,** 358-366.

Marennikova, S. S., Shelukhina, E. M. and Efremova, E. V. (1984). New outlook on the biology of cowpox virus. *Acta Virologica,* **28,** 437-444.

Marennikova, S. S., Shelukhina, E. M. and Fimina, V. A. (1978). Pox infection in white rats. *Laboratory Animals,* **12,** 33-36.

Marková, J., Machačová, T., Bártová, E., Sedlák, K., Budíková, M., Silvestre, P., Laricchiuta, P., Russo, M. and Veneziano, V. (2019). *Toxoplasma gondii*,*Neospora caninum*and*Encephalitozoon cuniculi*in animals from captivity (zoo and circus animals). *Journal of Eukaryotic Microbiology,* **66,** 442-446.

Martina, B. E. E., van Doornum, G., Dorrestein, G. M., Niesters, H. G. M., Stittelaar, K. J., Wolters, M. A. B. I., van Bolhuis, H. G. H. and Osterhaus, A. D. M. E. (2006). Cowpox virus transmission from rats to monkeys, the Netherlands. *Emerging Infectious Diseases,* **12,** 1005-1007.

Mätz-Rensing, K., Ellerbrok, H., Ehlers, B., Pauli, G., Floto, A., Alex, M., Czerny, C.-P. and Kaup, F.-J. (2006). Fatal Poxvirus Outbreak in a Colony of New World Monkeys. *Veterinary Pathology,* **43,** 212-218.

McInerney, J., Papasouliotis, K., Simpson, K., English, K., Cook, S., Milne, E. and Gunn-Moore, D. A. (2016). Pulmonary cowpox in cats: five cases. *Journal of Feline Medicine and Surgery,* **18,** 518-525.

Mellor, D., Hunt, S. and Gusset, M. (2015). Caring for wildlife: The World Zoo and Aquarium Animal Welfare Strategy. WAZA Executive Office, Gland, Switzerland.

Meyer, H., Osterrieder, N. and Czerny, C. P. (1994). Identification of binding sites for neutralizing monoclonal antibodies on the 14-kDa fusion protein of orthopox viruses. *Virology,* **200,** 778-783.

Meyer, H., Ropp, S. L. and Esposito, J. J. (1997). Gene for A-type inclusion body protein is useful for a polymerase chain reaction assay to differentiate orthopoxviruses. *Journal of Virological Methods,* **64,** 217-221.

Miller-Keane, M. O. T. (2006). Miller-Keane Encyclopedia and Dictionary of Medicine, Nursing, and Allied Health. Saunders, Philadelphia, pp. 2304.

Morgan, C. (1976). Vaccinia virus reexamined: development and release. *Virology,* **73,** 43-58.

Moss, B. (1990). Regulation of vaccinia virus transcription. *Annual Review of Biochemistry,* **59,** 661-688.

Moss, B. (2007). *Poxviridae*: the viruses and their replication. In: *Fields Virology*, D. Knipe and P. Howley, Eds, Vol. II, Lippincott Williams & Wilkins, Philadelphia, pp. 2906-2945.

Müller, H. K., Wittek, R., Schaffner, W., Schümperli, D., Menna, A. and Wyler, R. (1978). Comparison of five poxvirus genomes by analysis with restriction endonucleases*Hind*III,*Bam*I and*Eco*RI. *Journal of General Virology,* **38,** 135-147.

Murphy, E. G., Williams, N. J., Jennings, D., Chantrey, J., Verin, R., Grierson, S., McElhinney, L. M. and Bennett, M. (2019). First detection of Hepatitis E virus (Orthohepevirus C) in wild brown rats (*Rattus norvegicus*) from Great Britain. *Zoonoses and public health,* **66,** 686-694.

Nemat, A., Ali, Z., Ahmad, S., Sikander, S. K. and Hussain, Z. (2015). Study of disease records of zoo animals in Lahore zoo, Pakistan. *Journal of Animal and Plant Sciences,* **25,** 483-492.

Nitsche, A., Kurth, A. and Pauli, G. (2007). Viremia in human cowpox virus infection. *Journal of Clinical Virology,* **40,** 160-162.

Nitsche, A. and Pauli, G. (2007). Sporadic human cases of cowpox in Germany. *Euro Surveillance,* **12,** E070419.070413.

Oldal, M., Sironen, T., Henttonen, H., Vapalahti, O., Madai, M., Horváth, G., Dallos, B., Kutas, A., Földes, F., Kemenesi, G., Németh, V., Bányai, K. and Jakab, F. (2015). Serologic survey of orthopoxvirus infection among rodents in hungary. *Vector Borne And Zoonotic Diseases (Larchmont, N.Y.),* **15,** 317-322.

Parliament of the United Kingdom. (1974). Health and Safety at Work etc. Act 1974, UK Public General Acts, Ed, United Kingdom.

Pastoret, P.-P., Bennett, M., Brochier, B. and Akakpo, A. (2000). Animals, Public Health and the Example of Cowpox. *Revue Scientifique et Technique. Office International des Epizooties,* **19,** 23-32.

Paula, N. F. d., Dutra, K. S., Oliveira, A. R. d., Santos, D. O. d., Rocha, C. E. V., Vitor, R. W. d. A., Tinoco, H. P., Costa, M. E. L. T. d., Paixão, T. A. d. and Santos, R. L. (2020). Host range and susceptibility to Toxoplasma gondii infection in captive neotropical and Old−world primates. *Journal of Medical Primatology,* **49,** 202.

Payne, L. G. and Kristenson, K. (1979). Mechanism of vaccinia virus release and its specific inhibition by N1-isonicotinoyl-N2-3-methyl-4-chlorobenzoylhydrazine. *Journal of Virology,* **32,** 614-622.

Pelkonen, P. M., Tarvainen, K., Hynninen, A., Kallio, E. R. K., Henttonen, H., Palva, A., Vaheri, A. and Vapalahti, O. (2003). Cowpox with severe generalized eruption, Finland. *Emerging Infectious Diseases,* **9,** 1458-1461.

Pfeffer, M., Pfleghaar, S., Bomhard, D. v., Kaaden, O. R. and Meyer, H. (2002). Retrospective investigation of feline cowpox in Germany. *Veterinary Record,* **150,** 50-51.

Pilaski, J. and Jacoby, F. (1993). Die Kuhpocken-Erkrankungen der Zootiere. *Verh ber Erkrg Zootiere,* **35,** 39-50.

Postma, B. H., Diepersloot, R. J., Niessen, G. J. and Droog, R. P. (1991). Cowpox-virus-like infection associated with rat bite. *Lancet,* **337,** 733-734.

Prkno, A., Hoffman, D., Goerigk, D., Kaiser, M., van Maanen, A. C. F., Jeske, K., Jenckel, M., Pfaff, F., Vahlenkamp, T. W., Beer, M., Ulrich, R. G., Starke, A. and Pfeffer, M. (2017). Epidemiological investigations of four cowpox virus outbreaks in alpaca herds, Germany. *Viruses,* **9,** 344.

Public Health England. (2019). *List of zoonotic diseases*. https://www.gov.uk/government/publications/list-of-zoonotic-diseases/list-of-zoonotic-diseases. Accessed on: 06/08/2020.

Ropp, S. L., Jin, Q., Knight, J. C., Massung, R. F. and Esposito, J. J. (1995). PCR strategy for identification and differentiation of small pox and other orthopoxviruses. *Journal of Clinical Microbiology,* **33,** 2069-2076.

Schaudien, D., Meyer, H., Grunwald, D., Janssen, H. and Wohlsein, P. (2007). Concurrent infection of a cat with cowpox virus and feline parvovirus. *Journal of Comparative Pathology,* **137,** 151-154.

Schmelz, M., Sodeik, B., Ericsson, M., Wolffe, E. J., Shida, H., Hiller, G. and Griffiths, G. (1994). Assembly of vaccinia virus: the second wrapping cisterna is derived from the trans Golgi network. *Journal of Virology,* **68,** 130-147.

Schmiedeknecht, G., Eickmann, M., Köhler, K., Herden, C. E., Kolesnikova, L., Förster, C., Burkhardt, E. H., König, M., Thiel, M. and Reinacher, M. (2010). Fatal Cowpox Virus Infection in Captive Banded Mongooses (Mungos mungo). *Veterinary Pathology,* **47,** 547-552.

Schöniger, S., Chan, D. L., Hollinshead, M., Humm, K., Smith, G. L. and Beard, P. M. (2007). Cowpox virus pneumonia in a domestic cat in Great Britain. *Veterinary Record,* **160,** 522-523.

Schulze, C., Alex, M., Schirrmeier, H., Hlinak, A., Engelhardt, A., Koschinski, B., Beyreiss, B., Hoffmann, M. and Czerny, C. P. (2007). Generalized fatal cowpox virus infection in a cat with transmission to a human contact case. *Zoonoses and Public Health,* **54,** 31-37.

Schüppel, K., Menger, S., Eulenberger, K., Bernhard, A. and Pilaski, J. (1997). Kuhpockeninfektion bei Alpakas (*Lama glama pacos*). In: *Internationalen Symposiums über die Erkrankungen der Zoo- und Wildtiere*, Zürich, Schweiz, pp. 259-265.

Schwarzer, H., Kurth, A., Hermel, M. and Plange, N. (2013). Severe ulcerative keratitis in ocular cowpox infection. *Graefe's Archive for Clinical and Experimental Ophthalmology,* **251,** 1451-1452.

Shchelkunov, S., Marennikova, S. and Moyer, R. (2005). *Orthopoxviruses pathogenic for humans*. Springer, New Yolk, pp. 425.

Shchelkunov, S. N., Shcherbakov, D. N., Maksyutov, R. A. and Gavrilova, E. V. (2011). Species-specific identification of variola, monkeypox, cowpox, and vaccinia viruses by multiplex real-time PCR assay. *Journal of Virological Methods,* **175,** 163-169.

Shi, S. R., Key, M. E. and Kalra, K. L. (1991). Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical

staining based on microwave oven heating of tissue sections. *Journal of Histochemistry and Cytochemistry,* **39,** 741-748.

Silva, N. I. O., de Oliveira, J. S., Kroon, E. G., Trindade, G. S. and Drumond, B. P. (2020). Here, there, and everywhere: the wide host range and geographic distribution of zoonotic orthopoxviruses. *Viruses,* **13**.

Smith, K. C., Bennett, M. and Garrett, D. C. (1999). Skin lesions caused by orthopoxvirus infection in a dog. *Journal of Small Animal Practice,* **40,** 495-497.

Sodeik, B., Doms, R. W., Ericsson, M., Hiller, G., Machamer, C. E., Van't Hof, W., Van Meer, G., Moss, B. and Griffiths, G. (1993). Assembly of vaccinia virus: Role of the intermediate compartment between the endoplasmic reticulum and the Golgi stacks. *Journal of Cell Biology,* **121,** 521-542.

Stagegaard, J., Kurth, A., Stern, D., Dabrowski, P. W., Pocknell, A., Nitsche, A. and Schrick, L. (2017). Seasonal recurrence of cowpox virus outbreaks in captive cheetahs (*Acinonyx jubatus*). *Plos One,* **12,** e0187089.

Stewart, K. J., White, M. I., Telfer, S. and Bown, K. J. (2000). Cowpox infection: Not yet consigned to history. *British Journal of Plastic Surgery,* **53,** 348-350.

Stidworthy, M. (2010). Rodent-associated infections in zoo animals. *British Veterinary Zoological Society Spring Meeting*, Torquay, United Kingdom, 24th-25th April 2010, pp. 30-32.

Strenger, V., Müller, M., Richter, S., Revilla-Fernandez, S., Nitsche, A., Klee, S. R., Ellerbrok, H. and Zenz, W. (2009). A 17-year-old girl with a black eschar. *Clinical Infectious Diseases,* **48,** 91-92,133-134.

Taylor, C. (2014). Immunohistochemistry in surgical pathology: principles and practice. In: *Histopathology: methods and protocols*, C. Day, Ed, Humana Press, New York, pp. 81-109.

Tolonen, N., Doglio, L., Schleich, S. and Locker, J. K. (2001). Vaccinia virus DNA replication occurs in endoplasmic reticulum-enclosed cytoplasmic mini-nuclei. *Molecular Biology of the Cell,* **12,** 2031-2046.

Tsanava, S. A., Sakvarelidze, L. A. and Shelukhina, E. M. (1989). Serologic survey of wild rodents in Georgia for antibodies to orthopoxviruses. *Acta Virologica,* **33,** 91.

von Bomhard, W., Mauldin, E. A., Breuer, W., Pfleghaar, S. and Nitsche, A. (2011). Localized cowpox infection in a 5-month-old Rottweiler. *Veterinary Dermatology,* **22,** 111-114.

Vorou, R. M., Papavassiliou, V. G. and Pierroutsakos, I. N. (2008). Cowpox virus infection: an emerging health threat. *Current Opinion in Infectious Diseases,* **21,** 153-156.

Weber, S., Jeske, K., Beer, M., Hoffmann, D., Ulrich, R. G., Imholt, C. and Jacob, J. (2020). *In vivo*characterization of a bank vole-derived cowpox virus isolate in natural hosts and the rat model. *Viruses,* **12,** 237.

Webster, J. P. and MacDonald, D. W. (1995). Parasites of wild brown rats (*Rattus norvegicus*) on UK farms. *Parasitology,* **111,** 247-255.

Wendt, R., Tittelbach, J., Schrick, L., Kellner, N., Kalbitz, S., Ruehe, B., Michel, J., Schliemann, S., Elsner, P., Lübbert, C. and Nitsche, A. (2021). Generalized cowpox virus infection in an immunosuppressed patient. *International Journal of Infectious Diseases,* **106,** 276-278.

Wisser, J., Rudolph, M., Frolich, K., Pilaski, J., Strauss, G., Meyer, H., Burck, G. and Truyen, U. (2001). Cowpox virus infection causing stillbirth in an Asian elephant (*Elephas maximus*). *Veterinary Record,* **149,** 244.

Wolfs, T. F. W., Wagenaar, J. A., Niesters, H. G. M. and Osterhaus, A. D. M. E. (2002). Rat-to-human transmission of cowpox infection. *Emerging Infectious Diseases,* **8,** 1495-1496.

Yael, M., Nathan, Z., Ilana, S., Abraham, M. and Michael, G. R. (2010). Vaccinia-like cytoplasmic replication of the giant Mimivirus. *Proceedings of the National Academy of Sciences of the United States of America,* **107,** 5978.

Żaba, R., Jałowska, M., Kowalczyk, M. J., Bowszyc-Dmochowska, M., Adamski, Z. and Szkaradkiewicz, A. (2017). Cowpox virus infection in a child after contact with a domestic cat: a case report. *New Microbiologica,* **40,** 148-150.

Zwart, P., Gispen, R. and Peters, J. C. (1971). Cowpox in Okapis*Okapia johnstoni*atRotterdam Zoo. *British Veterinary Journal,* **127,** 20-24.